Unproductive ribosome stalling in eubacteria is resolved by the actions of SmpB protein and transfer messenger (tm) RNA. We examined the functional significance of保守 sequenced regions of SmpB and tmRNA to the trans-translation process. Our investigations reveal that the N-terminal 20 residues of SmpB, which are located near the ribosomal decoding center, are dispensable for all known SmpB activities. In contrast, a set of conserved residues that reside at the junction between the tmRNA-binding core and the C-terminal tail of SmpB play an important role in tmRNA accommodation. Our data suggest that the highly conserved glycine 132 acts as a flexible hinge that enables movement of the C-terminal tail, thus permitting proper positioning and establishment of the tmRNA open reading frame (ORF) as the surrogate template. To gain further insights into the function of the SmpB C-terminal tail, we examined the tagging activity of hybrid variants of tmRNA and the SmpB protein, in which the tmRNA ORF or the SmpB C-terminal tail was substituted with the equivalent but highly divergent SmpB protein, in which the tmRNA ORF or the SmpB C-terminal tail was substituted with the equivalent but highly divergent SmpB sequence from Francisella tularensis. We observed that the hybrid tmRNA was active but resulted in less accurate selection of the resume codon. Cognate hybrid SmpB was necessary to restore activity. Furthermore, accurate tagging was observed when the identity of the resume codon was reverted from GGC to GCA. Taken together, these data suggest that the engagement of the tmRNA ORF and the selection of the correct translation resumption point are distinct activities that are influenced by independent tmRNA and SmpB determinants.

Protein biosynthesis is a complex molecular process orchestrated by the ribosome and a multitude of other RNA and protein factors. The canonical events of translation, from initiation to termination, have been studied in great detail over the past few decades (1). These explorations have led to an improved structural and mechanistic understanding of how most translation events occur in bacteria. However, much less is known about how bacterial ribosomes accomplish more specialized tasks such as initiation at leaderless mRNAs (2), programmed bypass on certain phage genes (3), and ribosome rescue by tmRNA (4–12).

The process by which tmRNA and its protein partner SmpB rescue stalled ribosomes is known as trans-translation. During this process, tmRNA, aminocylated with alanine and bound to a single molecule of SmpB (13) and EF-Tu-GTP (14, 15), enters the A-site of the stalled ribosome and facilitates the transfer of the nascent polypeptide to its tRNA-like domain. Unlike standard tRNAs, tmRNA does not contain an anti-codon loop. In place of the anti-codon loop, tmRNA contains a series of pseudoknots that are interrupted by a short open reading frame (ORF) in a region known as the mRNA-like domain. Concomitant with this initial transpeptidation, the SmpB-tmRNA rescue complex is able to coax the ribosome to resume translation on the tmRNA ORF and disengage the defective mRNA (7).

There are numerous consequences to this template switching action promoted by tmRNA and SmpB. The presence of an in-frame stop codon in the tmRNA ORF promotes efficient translation termination and recycling of the ribosomal subunits. The tmRNA peptide tag that is co-translationally appended to the nascent polypeptide contains recognition signals for cellular proteases (16–20). Thus, the potentially detrimental nascent polypeptide is marked for directed proteolysis. Finally, RNase R is engaged in an SmpB-tmRNA-dependent manner to degrade the defective mRNA (21, 22).

Determinants for various aspects of the trans-translation process have been identified in both tmRNA and SmpB. For instance, degradation of the defective mRNA is dependent on residues in the mRNA-like domain of tmRNA, specifically stop codon proximal residues in helix 5 (22, 23). Additionally, mutations that destabilize helix 5 lower the tagging activity of
SmpB C Terminus and Hinge Are Required for Ribosome Rescue

tmRNA (24, 25). SmpB has a conserved RNA binding surface that interacts with the tmRNA-like domain during delivery of tmRNA to the ribosome (13, 26–29). SmpB plays an additional critical role in trans-translation, beyond delivery of tmRNA to the stalled ribosome. SmpB variants that lack the C-terminal tail support delivery of tmRNA to stalled ribosomes but are unable to assist in subsequent steps of trans-translation (27). A number of genetic and structural studies have suggested that the C-terminal tail of SmpB is necessary for accommodation and transpeptidation and ultimately the establishment and selection of the tmRNA ORF (27, 30, 31). This task might be accomplished by direct interactions between the C-terminal tail of SmpB and the mRNA-like domain of tmRNA (32–34). Furthermore, recent structural studies have suggested that SmpB specifically recognizes stalled ribosomes through interactions between its C-terminal tail and the ribosomal mRNA channel (28). The C-terminal tail of SmpB is thus postulated to act as an mRNA mimic, filling the otherwise empty mRNA channel of ribosomes stalled at the 3' end of a nonstop mRNA.

Template switching is a pivotal step during trans-translation. There are at least two problems that tmRNA must overcome to accomplish this task. First, the mRNA-like domain of tmRNA needs to be loaded into the A-site and mRNA channel of the 30 S subunit. For this to occur, some structural rearrangements are necessary to allow tmRNA access to the mRNA channel, which normally adopts a closed tunnel-like conformation around the mRNA template (28, 35, 36). It is not clear how SmpB and tmRNA interact to promote these conformational changes in the ribosome. Furthermore, access to the mRNA channel alone is not sufficient for proper ribosome rescue by tmRNA. To append the intended destabilizing tag sequence to the nascent peptide, the ribosome must select, or be directed to, the correct codon upon which to resume translation. Normal initiation factors and Shine-Dalgaro/anti-Shine-Dalgaro interactions are a priori absent during this process, raising the question as to how accurate reading frame selection is achieved during trans-translation (1).

In this study, we sought to gain new insights into how accurate reading frame selection is achieved during trans-translation and what SmpB and tmRNA determinants are required for accurate establishment of the tmRNA ORF as a surrogate mRNA. More specifically, we wished to elucidate the role of highly conserved sequence motifs of the SmpB protein in promoting the establishment and selection of the tmRNA reading frame. To achieve this aim, we constructed and evaluated SmpB variants carrying amino acid substitutions in highly conserved motifs in the N- and C-terminal tails of the protein. We also engineered hybrid versions of SmpB and tmRNA, from genetically distant bacterial species, in which the mRNA-like domain of tmRNA or the C-terminal tail of SmpB from Yersinia pseudotuberculosis was substituted with the equivalent sequences from Francisella tularensis. Our investigations revealed that the highly conserved N-terminal tail of SmpB is dispensable for all known trans-translation related activities of the protein, whereas the highly conserved Lys-131–Gly-132–Lys-133–Lys-134 region, which links the tmRNA binding core and the C-terminal tail of the protein, plays a distinct but crucial role in trans-translation. Our investigation of hybrid constructs revealed that although the tmRNA ORF hybrid could robustly engage the ribosome, it suffered defects in accurate selection of the resume codon. Further modification of distal portion of the ORF in the hybrid tmRNA led to a dependence on the cognate SmpB C-terminal tail hybrid for activity. Finally, we observed an SmpB-dependent preference for the identity of the translation resume signals on the mRNA-like domain of tmRNA.

EXPERIMENTAL PROCEDURES

SmpB Gly132X Mutagenesis and Screening—To generate a pool of SmpB variants at amino acid position 132 of SmpB, plasmid pET28BAH6 containing Escherichia coli SmpB and a tmRNA variant encoding a tag containing six histidines, tmRNAH6, were mutagenized using the QuikChange mutagenesis kit with the following primers: Gly132-random reverse (5'-GGT-TATCGTGCTGTTCCTTNNNNTGGCGACGCCG-3') and Gly132-random forward (5'-CGGCGTCGCCAAANNNNAA-GAAACAGCAGCATAAAC-3'), where NNN is the randomized Gly132 codon. We generated a pool of 256 SmpBgly132X variants in E. coli strain W3110 ΔsmpBssrA. Clones were screened for susceptibility to lysis by αimmP22 c2-5dis hybrid phage as described previously (21). Briefly, 2 µl of phage were spotted onto Luria-Bertani broth (LB) agar plates and allowed to adsorb. The various SmpB Gly132X clones in E. coli strain W3110 ΔsmpBssrA were streaked through the phage spot and incubated overnight at 37 °C. The SmpB gene of pET28BAH6 for all clones able to support lysis by phage was sequenced, and the identity of amino acids at position 132 was determined. Several clones that were not able to support the lytic growth of αimmP22 c2-5dis hybrid phage were also sequenced and characterized.

tmRNAH6 Tagging Assay—The endogenous tagging assay protocol used is a modification of Sundermeier et al. (37). Briefly, 50 ml of ssrA::cat, ΔsmpB (DE3), and ΔsmpBssrA (DE3) with pETBAH6 were grown in LB to an OD600 of 0.8–0.9. The ΔsmpB (DE3) and ΔsmpBssrA (DE3) cultures were held under constant induction using 10 µl IPTG. Cells were harvested and resuspended in 1 ml of lysis buffer (8 M urea, 100 mM potassium phosphate, pH 8, 10 mM Tris, pH 8, and 5 mM β-mercaptoethanol) and lysed by rocking at room temperature for 1 h. Cell debris was removed by centrifugation, and the cleared supernatant was added to 100 µl of Ni2+-NTA-agarose resin (Qiagen). The specific binding of His6-tagged protein to the resin was allowed to proceed for 1 h at room temperature. Resin sample slurries were applied to a mini-chromatography column (Bio-Rad) and washed 4 times with 1 ml of lysis buffer. Bound proteins were eluted in 150 µl of elution buffer (8 M urea, 100 mM acetic acid, and 20 mM β-mercaptoethanol), resolved by electrophoresis on a 15% Tris–Tricine gel, and transferred to polyvinylidene difluoride (PVDF) membranes. The resolved His6-tagged proteins were probed with a primary mouse anti-His6 antibody (Covance) and a goat anti-mouse IR800 dye-conjugated secondary antibody (Molecular Probes), detected using the Odyssey Infrared Imaging System, and quantified using the Odyssey data analysis software (LI-COR).

λ-Nonstop Reporter Expression—E. coli strain W3110 ΔsmpBssrA (DE3), which lacks the necessary components for trans-translation, was transformed with pKW846. This plasmid contains a T7-driven truncated variant of the N-terminal
domain of the λcl repressor (hereafter λ-cl), followed by the trpA gene transcriptional terminator (trpA). This strain was then transformed with an additional plasmid, encoding the indicated variants of SmpB and tmRNA under the control of their native promoter. These strains were grown in LB broth to an OD_{600} of 0.6, and the λ-cl reporter gene expression was induced using 1 mM IPTG. After 3 h of induction, an equal number of cells were harvested and resuspended in 150 μl of gel loading dye containing 8 M urea.

Ribosome Association Assay—To prepare tight-coupled 70 S ribosomes, a 750-ml culture of W3110 harboring ΔsmpBΔsrA (DE3) containing plasmid pET28 BA^{146} or pET28 BA^{146} with the specified SmpB amino acid substitutions variants was grown in LB containing 1 μM IPTG and 50 μg/ml kanamycin to an OD_{600} of 0.800–1.00. Cells were harvested by centrifugation and stored at −80 °C. Cell pellets were resuspended in buffer A (20 mM Tris, pH 7.5, 500 mM NH_{4}Cl, 10 mM MgCl_{2}, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 units/ml SuperASE In (Ambion)) and lysed by sonication with three 30-s pulses on ice, with the addition of 0.1 ml of 100 mM PMSF and a 1-min storage on ice after each pulse. Lysates were centrifuged at 30,000 × g for 30 min in an SS-34 rotor (Sorvall). Supernatants were transferred to new tubes and centrifuged again at 30,000 × g for 30 min. Typically, a 19-ml aliquot of the supernatant was layered onto a 19-ml 32% sucrose cushion in buffer B (20 mM Tris, pH 7.5, 500 mM NH_{4}Cl, 10 mM MgCl_{2}, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 units/ml SuperASE In) and centrifuged at 85,000 × g for 22 h. The pellet containing tight-coupled ribosomes was washed twice with 5 ml of cold buffer B. Tight-coupled 70 S ribosomes were resuspended in 500 μl of resuspension buffer (20 mM Tris, pH 7.5, 100 mM NH_{4}Cl, 10 mM MgCl_{2}, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 units/ml SuperASE In) and subjected to centrifugation for 10 min, and loaded on a Mono S ion exchange column (GE Healthcare). A gradient of 50–850 mM KCl was developed over 20 column volumes to isolate the SmpB protein. SmpB protein, with greater than 95% purity, eluted at ~450–500 mM KCl under these conditions. Protein concentrations were determined by absorbance at 280 nm, using an extinction coefficient of 29,575 M^{-1} cm^{-1} for E. coli SmpB^{W7}. Protein aliquots were stored at −80 °C.

Western Blot Analysis—Samples from λ-cl nonstop expression experiments or endogenous tagging experiments were resolved overnight on a 20 or 15% Tris-Tricine-SDS-polyacrylamide gel. Resolved proteins were transferred to PVDF membranes. The membrane-bound proteins were probed with primary antibodies to the His_{6} or the FLAG epitope and secondary antibody conjugated to a fluorescent dye, detected using the Odyssey infrared imaging system, and quantified using the Odyssey data analysis software (LI-COR and Image).

RESULTS

SmpB Glycine 132 Is Important for trans-Translation—Previous studies have established that the SmpB protein is a requisite factor in the tmRNA-mediated ribosome rescue system (7, 9, 10). SmpB binds specifically and with high affinity to the RNA-like domain of tmRNA and is necessary for the recruitment and stable association of the SmpB-tmRNA complex with stalled ribosomes (15, 30). SmpB is also required for a novel function following ribosome association but prior to the addition of the tmRNA alanine charge (27). We sought to elucidate the specific roles of SmpB by identifying amino acid residues (Fig. 1) that are essential for various stages of the trans-translation process. To begin, we performed a random mutagenesis screen of SmpB protein. We subjected a plasmid harboring the E. coli smpB and srrA genes (pET28BA) to hydroxylamine mutagenesis and transformed the mutagenized plasmids into an smpB-ssrA-deficient strain. Clones generated from this mutagenesis were screened for their ability to support growth of ximmP22 c2-5dis, a λ-P22 hybrid phage (8, 15, 38) that requires active trans-translation for lytic growth in E. coli (Fig. 2A). Using this assay, we recovered clones that were unable to support phage growth. We sequenced a number of fully and partially defective SmpB variants from these clones. We concentrated our initial efforts on SmpB^{G132D}, a variant where the glycine residue at position 132 (Gly-132) was substituted with an aspartic acid residue. The SmpB^{G132D} variant exhibited the most severe defect in supporting the lytic growth of ximmP22 c2-5dis hybrid phage (Fig. 2B). This variant displayed similar levels of protein expression and solubility as wild-type SmpB (SmpB^{W7}), indicating that the mutation did not impact the availability of properly folded SmpB protein in the cell. The observed defect in hybrid phage growth, combined with the high degree of sequence conservation at this position (Fig. 1A), suggested that Gly-132 supports a critical and novel SmpB function in the trans-translation process.

SmpB C Terminus and Hinge Are Required for Ribosome Rescue

Purification of SmpB Protein—Bacterial strain BL21 (DE3)/pLysS (Stratagene) was transformed with plasmid pET28 BAH6 or pET28 BAH6 with the specified SmpB amino acid substitutions variants was grown in LB containing 1 μM IPTG and 50 μg/ml kanamycin to an OD_{600} of 0.800–1.00. Cells were harvested by centrifugation and stored at −80 °C. Cell pellets were resuspended in buffer A (20 mM Tris, pH 7.5, 500 mM NH_{4}Cl, 10 mM MgCl_{2}, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 units/ml SuperASE In (Ambion)) and lysed by sonication with three 30-s pulses on ice, with the addition of 0.1 ml of 100 mM PMSF and a 1-min storage on ice after each pulse. Lysates were centrifuged at 30,000 × g for 30 min in an SS-34 rotor (Sorvall). Supernatants were transferred to new tubes and centrifuged again at 30,000 × g for 30 min. Typically, a 19-ml aliquot of the supernatant was layered onto a 19-ml 32% sucrose cushion in buffer B (20 mM Tris, pH 7.5, 500 mM NH_{4}Cl, 10 mM MgCl_{2}, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 units/ml SuperASE In) and centrifuged at 85,000 × g for 22 h. The pellet containing tight-coupled ribosomes was washed twice with 5 ml of cold buffer B. Tight-coupled 70 S ribosomes were resuspended in 500 μl of resuspension buffer (20 mM Tris, pH 7.5, 100 mM NH_{4}Cl, 10 mM MgCl_{2}, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 10 units/ml SuperASE In) and subjected to Western and Northern blot analyses. RNA for Northern blot analysis was extracted with Tri-LS reagent (Molecular Research), and equal amounts of RNA were loaded onto 1% formaldehyde-agarose gels, transferred to Hi-Blot nylon membrane (Amersham Biosciences), and probed with a psoralen-biotin (Ambion)-labeled full-length tmRNA oligonucleotide probe. For Western blot analysis, equal numbers of ribosomes were loaded per lane and resolved by electrophoresis on 15% denaturing Tris-Tricine gels. Western blots were probed with rabbit polyclonal antibodies raised against purified SmpB protein and a secondary IR800 dye-conjugated secondary anti-rabbit antibody (Molecular Probes).

Purification of SmpB Protein—Bacterial strain BL21 (DE3)/pLysS (Stratagene) was transformed with plasmid pET28 BA^{146} harboring SmpB^{W7} or one of the alanine substitution variants: SmpB^{ΔN17A/A18A/R19A}, SmpB^{K131A/K133A/K134A}, or SmpB^{G132D}. Typically, cells were grown in 3 liters of LB at 37 °C to an OD_{600} of 0.500 and induced with 1 mM IPTG for 3 h. Cells were harvested and resuspended in lysis buffer (1 mM NH_{4}Cl, 150 mM KCl, 50 mM Tris, pH 8.0, 2 mM β-mercaptoethanol, 20 mM imidazole) and lysed by sonication. Cell lysates were centrifuged for 1 h at 30,000 × g. Supernatants were mixed with 2 ml of Ni^{2+}-NTA resin (Qiagen) pre-equilibrated in lysis buffer and permitted to bind for 1 h at 4 °C with continuous rocking. The Ni^{2+}-NTA resin sample slurry was applied to a chromatography column and washed three times with 30 ml of lysis buffer. Proteins were eluted in three steps with 2 ml of elution buffer (150 mM KCl, 50 mM Tris, pH 8.0, 200 mM imidazole, 20 mM β-mercaptoethanol). The eluate was diluted to 50 ml in FPLC buffer A (50 mM KCl, 50 mM HEPES, pH 7.5, 5 mM MgCl_{2}, 2 mM β-mercaptoethanol), subjected to centrifugation for 10 min, and loaded onto a Mono S ion exchange column (GE Healthcare). A gradient of 50–850 mM KCl was developed over 20 column volumes to isolate the SmpB protein. SmpB protein, with greater than 95% purity, eluted at ~450–500 mM KCl under these conditions. Protein concentrations were determined by absorbance at 280 nm, using an extinction coefficient of 29,575 M^{-1} cm^{-1} for E. coli SmpB^{W7}. Protein aliquots were stored at −80 °C.

Western Blot Analysis—Samples from λ-cl nonstop expression experiments or endogenous tagging experiments were resolved overnight on a 20 or 15% Tris-Tricine-SDS-polyacrylamide gel. Resolved proteins were transferred to PVDF membranes. The membrane-bound proteins were probed with primary antibodies to the His_{6} or the FLAG epitope and secondary antibody conjugated to a fluorescent dye, detected using the Odyssey infrared imaging system, and quantified using the Odyssey data analysis software (LI-COR and Image).
**SmpB Glycine 132 Can Be Substituted by Select Small Amino Acids**—To gain further insight into the role of Gly-132, we sought to identify amino acid residues that could be functionally substituted at this position. To this end, we used PCR mutagenesis to randomize the Gly-132 codon in a plasmid-borne copy of *smpB*. We screened 256 clones for their ability to support lytic growth of the /H9261 imm /P22 c2-5dis hybrid phage. Forty-five clones that could fully or partially support phage growth (complementing clones), as well as 12 noncomplementing clones, were sequenced, and the amino acid codons at position 132 were determined. This analysis revealed that the majority of the complementing clones, 33 out of 45, encoded for glycine at this position. Among the remaining complementing clones, alanine, histidine, phenylalanine, and arginine could either fully or partially support phage growth (complementing clones), as well as 12 noncomplementing clones, were sequenced, and the amino acid codons at position 132 were determined. This analysis revealed that the majority of the complementing clones, 33 out of 45, encoded for glycine at this position. Among the remaining complementing clones, alanine, histidine, phenylalanine, and arginine could either fully or partially support phage growth, suggesting that these residues could be exchanged for Gly-132 (Table 1). Substitution with a proline, which occurs naturally in some species of *Mycoplasma*, displayed an intermediate level of activity in supporting hybrid phage growth. In contrast, leucine, valine, threonine, serine, aspartate, glutamate, and glutamine substitutions were unable to support growth of the hybrid phage, indicating that they disrupted a critical SmpB function (Table 1).

Although the *aimmp22 c2-5dis* phage assay is a good initial indicator of SmpB protein function, it does not provide a quantitative measure of the level of endogenous tmRNA tagging activity. Therefore, to verify the results obtained with the hybrid phage assay, we analyzed the SmpB Gly-132 variants for their ability to support tmRNA-mediated ribosome rescue and tagging of native substrates. To detect endogenous tagging events, we utilized a tmRNA variant (tmRNA<sup>H6</sup>) in which the last six residues of the tag were substituted with the His<sub>6</sub> motif. The tmRNA<sup>H6</sup> variant permits tagged proteins to be purified by Ni<sup>2+</sup>-NTA affinity chromatography and visualized by Western blot analysis with anti-His<sub>6</sub> antibodies (37). The hybrid phage complementing clones were able to support tagging at or near wild-type levels (Table 1). Two exceptions were the variants SmpBG<sub>132F</sub> and SmpBG<sub>132R</sub>. These substitutions were able to support tagging activity but at substantially reduced levels compared with SmpB<sup>WT</sup>. Substitution of Gly-132 with proline, the other naturally occurring substitution at this position, was unable to fully complement the endogenous tagging activity. Instead, consistent with the phage growth phenotype, we observed an intermediate level of tagging with the SmpBG<sub>132P</sub> variant (Table 1).

We also analyzed the noncomplementing clones for their ability to support the tmRNA-mediated polypeptide tagging activity (Fig. 2 and Table 1). The SmpBG<sub>G132L</sub> and SmpBG<sub>G132E</sub> variants displayed an almost complete loss of endogenous pro-
tein tagging activity. The SmpB variants SmpB\textsubscript{G132V} and SmpB\textsubscript{G132T} were able to support endogenous tagging activity but at greatly reduced levels compared with SmpB\textsubscript{WT} (Fig. 2, C and D). The general trend of requiring smaller residues at this position suggested that this region might function as a fulcrum or hinge to enable accommodation of the tmRNA ORF as the surrogate template at an early stage of the translational process.

**Additional A-site Proximal SmpB Residues Impact tmRNA Tagging Activity**—The lysine residues Lys-131, Lys-133, and Lys-134 that are adjoining the nearly invariant Gly-132 also display strong sequence conservation among bacterial species, suggesting they might make important contributions to SmpB function (Fig. 1). We engineered single, double, and triple alanine substitution variants of these conserved residues and analyzed the propensity of the resulting SmpB variants in supporting ribosome association and endogenous substrate tagging activities. The SmpB\textsubscript{K131A/K133A} and SmpB\textsubscript{K131A/K134A} variants tagged endogenous proteins at levels similar to SmpB\textsubscript{WT} (Fig. 3A). The most defective double alanine substitution variant, SmpB\textsubscript{K133A/K134A}, displayed close to 30% reduction in tagging activity. The triple alanine substitution variant SmpB\textsubscript{K131A/K133A/K134A} had the most deleterious effect on endogenous tagging (Fig. 3B), with greater than 75% reduction in endogenous protein tagging activity. Importantly, none of the substitutions made in this region impacted the ability of SmpB to bind tmRNA and deliver it to ribosomes (Fig. 3C), indicating that these substitutions did not affect the native fold of the protein. These data suggest that the highly conserved basic residues in this region play an important role in translational process.

In addition to residues 131–134 in the SmpB hinge region, there exists a set of highly conserved residues in the N-terminal tail of the protein (Fig. 1) with close spatial proximity to the decoding center of the ribosomal A-site (28).
SmpB C Terminus and Hinge Are Required for Ribosome Rescue

TABLE 1

| SmpB variant | Tagging activity (% relative to wild type) | TimmP22 c2-5dis hybrid phage cross-streak assay |
|--------------|-------------------------------------------|---------------------------------------------|
| SmpB N17A    | 100                                       | ND                                          |
| SmpB K18A    | 100                                       | ND                                          |
| SmpB R19A    | 100                                       | ND                                          |
| SmpB N17A/K18A/R19A | 100                           | ND                                          |
| SmpB K18E/R19E | 100                           | ND                                          |
| SmpB N17A/K18E/R19E | 100                           | ND                                          |
| SmpB W14     | 100                                       | ND                                          |
| SmpB K131    | 100                                       | ND                                          |
| SmpB K132/K134 | 97                                          | ND                                          |
| SmpB K134    | 100                                       | ND                                          |
| SmpB K135    | 100                                       | ND                                          |
| SmpB G132A   | 94                                        | S                                           |
| SmpB G132D   | ND                                        | R                                           |
| SmpB G132E   | 2                                        | R                                           |
| SmpB G132F   | 62                                        | MS                                          |
| SmpB G132H   | 82                                        | R                                           |
| SmpB G132L   | 6                                        | MS                                          |
| SmpB G132P   | 77                                        | MS                                          |
| SmpB G132R   | 33                                        | S                                           |
| SmpB G132S   | 89                                        | R                                           |
| SmpB G132T   | 55                                        | R                                           |
| SmpB G132V   | 54                                        | R                                           |
| SmpB W1, tmRNA-His6 | N/A (100)                        | S                                           |
| SmpB W1, tmRNAF | 120                                      | ND                                          |
| SmpB FT, tmRNAF | 135                                      | ND                                          |
| SmpB W1, tmRNAF-T-H6 | 64                                     | R                                           |
| SmpB FT, tmRNAF-T-H6 | 119                                   | S                                           |
| SmpB W1, tmRNAF-T-H6 GCA resume | 76                                   | ND                                          |
| SmpB FT, tmRNAF-T-H6 GCA resume | 84                                   | ND                                          |
| SmpB FT, tmRNAF-T-H6 GCA resume | 131                                   | ND                                          |

Role through interactions with residues A1492 and A1943 in the decoding center (28). We sought to examine the functional significance of the conserved charged residues in this region. Toward this end, we constructed and analyzed several single, double, and triple alanine substitution variants at positions Asn-17, Lys-18, and Arg-19. Surprisingly, all alanine substitutions, including the SmpB N17A/K18A/R19A triple alanine substitution variant, exhibited near wild-type levels of tmRNA binding affinity and endogenous tagging activity (Fig. 3). The SmpB N17A/K18A/R19A variant was also fully competent in delivering tmRNA to stalled ribosomes (Fig. 3C). In fact, deletion of the N-terminal 20 residues of SmpB did not lead to a significant decrease in tmRNA tagging activity (Fig. 3D). This finding indicates that, despite their sequence conservation and positioning in a recent ribosome-bound structural model, these residues are not vital to the known trans-translation functions of SmpB protein.

Hybrid tmRNA Displays Robust yet Inaccurate Tagging—We next wished to examine the merits of the idea that the C-terminal tail of SmpB interacts with the ribosome and the mRNA-like domain of tmRNA and that these interactions play a crucial role in the accurate selection of the correct reading frame on tmRNA. We reasoned that if such functional interactions exist, they are likely to be species- and sequence-specific. Unlike the tRNA-like domain of tmRNA, its mRNA-like domain displays a greater degree of sequence divergence among different bacterial species. This is likely a consequence of the evolution of different tag sequences to suit the proteolytic requirements of each particular species (19). Likewise, the SmpB C-terminal tail (residues 133–161) features both highly conserved and highly divergent residues. As expected, the highly conserved tail residues play functionally significant roles (26, 27) and have recently been modeled to make potential contacts with the ribosome (28). These considerations suggest the intriguing possibility that the divergent residues in tmRNA and SmpB share species- and sequence-specific functional interactions during the establishment and selection of the tmRNA reading frame.

To explore the idea that there are functional interactions between the mRNA-like domain of tmRNA and the C-terminal tail of SmpB (amino acid residues 133–161), we created tmRNA ORF hybrids and SmpB C-terminal tail hybrids, such that the putative interacting regions were replaced with the corresponding yet highly divergent sequences from F. tularensis. We have shown that the SmpB-tmRNA ribosome rescue system is active in Y. pseudotuberculosis and F. tularensis (24, 25, 39), eliminating the possibility that the introduced sequences are inherently nonfunctional. A plasmid harboring SmpB-tmRNA from Y. pseudotuberculosis was altered to create hybrid constructs that contained the mRNA-like domain of F. tularensis tmRNA (SmpB-tmRNAF), the C-terminal tail (amino acid residues 133–157) of F. tularensis SmpB (SmpBFT-tmRNA), and the double hybrid containing the mRNA-like domain of F. tularensis tmRNA and the C-terminal tail of F. tularensis SmpB (SmpBFT-tmRNAF). These hybrids were assessed for their ability to append the much longer (23-amino acid residue) F. tularensis tmRNA tag to a reporter protein encoded by a λ-cl nonstop mRNA. Western blot analysis of the λ-cl reporter protein product revealed that the tmRNAF variant had robust tagging activity, even though it led to more than one species of tagged proteins (Fig. 4). Interestingly, both SmpB W1, as well as the SmpBFT hybrid, supported similar levels and patterns of tagging with the hybrid tmRNAF. Based on their apparent molecular weights, it was possible that the additional species of the tagged λ-cl reporter proteins were due to resumption of translation on alternate reading frames on the tmRNA ORF (see below). From these data, we inferred that sequence differences between Y. pseudotuberculosis and F. tularensis tmRNA were not important for establishment of the mRNA-like domain of tmRNA as a surrogate template, but they could play an important role in accurate selection of the correct reading frame within this new template.

tmRNAF-T-H6 Hybrid Is Dependent on Cognate SmpB C-terminal Tail Sequence for Activity—To facilitate the analysis of resume codon selection accuracy during tmRNA ribosome rescue, we modified the F. tularensis tmRNA hybrids so that the last six codons of the ORF encoded for six histidines (tmRNAF-T-H6). Some of the residues encoding these amino acids are found within the structurally important helix 5 of tmRNA. Therefore, the alterations necessary to encode the six histidines were matched with compensatory mutations to pre-
serve the structure of helix 5 (Fig. 5, A and B) (25). We found it surprising that the His6 substitution significantly decreased the tagging activity of the tmRNAFT-H6 hybrid (Fig. 5C), because His6 substitutions in the E. coli tmRNA do not result in such drastic reduction in tagging activity. Although the His6 substitution did not significantly alter the accuracy of resume codon selection, overall tagging activity decreased relative to the tmRNAFT hybrid (Fig. 5, D and E). More significantly, the tagging activity defect of the tmRNAFT-H6 variant could be rescued by co-expression of the SmpBFT hybrid (Fig. 6). Although the tmRNAFT was active irrespective of the identity of SmpB C-terminal tail, the activity of the tmRNAFT-H6 hybrid was now dependent on the presence of the cognate SmpB C-terminal tail (SmpBFT). Despite this increase in activity, the hybrid SmpBFT did not alter the pattern of multiple tagged species. When E. coli SmpBFT-tmRNAFT hybrids were made similar results were obtained, suggesting the phenotypes are not related to the use of Y. pseudotuberculosis SmpB and tmRNA (Fig. 7). Taken together, these data suggest a role for the SmpB C-terminal tail in reading frame establishment that is normally masked by other functionally important interactions involving the distal portion of the mRNA-like region of tmRNA. Furthermore, this role appears distinct from the selection of the proper resume codon on the tmRNA ORF.

FIGURE 3. Analysis of the activity of SmpB alanine substitution variants in the highly conserved Asn-17–Arg-19 and Lys-131–Lys-134 regions of SmpB. A, representative Western blot, developed with antibodies to the His6 epitope, displaying all of the endogenously tmRNAH6-tagged proteins. SmpB(N17A/R18A/R19A) is represented as SmpBAAA. The results shown are typical from a set of three independent experiments, all of which were quantified and averaged. B, bar graphs represent the mean ± S.D. of three independent tagging assays, displaying the level of tagging activity as compared with SmpBWT. SmpB(AA), a nonfunctional truncated version of SmpB, was used a negative control. C, Western blot analysis with anti-SmpB antibodies (top panel) displaying the amount of wild-type SmpB and select SmpB alanine substitution variants, associated with 70 S ribosomes in vivo. Northern blot analysis with a tmRNA-specific probe (middle panel) to detect 70 S ribosome-associated tmRNA. Ethidium bromide staining of the same gel as in the middle panel is shown to demonstrate that similar amounts of ribosomal RNA were loaded in each lane. The SmpB variant expressed in the cells from which the ribosomes were purified is indicated on the top panel. SmpB(N17A/R18A/R19A) is represented as SmpBAAA. D, representative Western blot, developed with antibodies to the His6 epitope, displaying all of the endogenously tagged proteins produced by a strain expressing SmpBWT, SmpB59, or SmpB(AA). SmpBWT is used as a positive control and SmpB59 as a negative control. Asn (N), Arg (R), and Lys (K).
Residues Near the Tip of Helix 5 Are Important for Engaging the tmRNA Reading Frame—The changes necessary to generate the His6 substitution have a clear effect on the tagging activity of hybrid tmRNA, making robust activity dependent on the presence of the cognate SmpB C-terminal tail. These changes encompass a region of ~40 nucleotides, including the compensatory mutations necessary to preserve the stability of helix 5. Within this region, almost half of the residues have been changed (Fig. 5B). Therefore, it was unclear which residues contributed to the observed tagging defects. To identify these determinants with better resolution, we generated a series of two-His tag variants, in which the 17th and 18th, 19th and 20th, or 21st and 22nd codons of the tmRNAFT ORF were substituted with histidine codons. Introduction of any of the two-His codon pairs, without additional mutations to preserve helix 5 stability, substantially diminished tagging activity. These data confirm the importance of the structural contributions of helix 5 during trans-translation (24, 25). When compensatory base pairing nucleotides were introduced alongside the two-His codon substitutions, a clear defect was observed only for the 21st and 22nd codon pair (Fig. 8A and B). Substitution at codon pairs 17–18 and 19–20 appear to have a much smaller effect when helix 5 stability is preserved. These data suggest that some or all of the seven nucleotides changed to encode histidine as the ultimate and penultimate codons are important for sequence-specific interactions during establishment of the tmRNA reading frame.

Hybrid tmRNA Variants Result in Inaccurate Reading Frame Establishment—When ribosomes are rescued by the hybrid tmRNA variants, either the tmRNAFT or the tmRNAFT-H6, we observed multiple tagged species of the λ-cl reporter protein (Fig. 8C). Based on the apparent molecular weight of these products, we postulated that the slowest migrating band was the λ-cl reporter appended with the full-length F. tularensis tag, whereas the other bands were the λ-cl reporter appended with −1 or +1 frame-shifted versions of the tag. In addition, Western blot analysis of the tmRNAFT-H6, using anti-His6 antibodies, was consistent with tagging in different reading frames (Fig. 8D) because only tagging in the correct reading frame produces a protein product with two His6 epitopes, one inherent to the reporter and the second arising from tagging in the correct reading frame by the tmRNAFT-H6 hybrid. Therefore, correctly tagged reporter products appear more intense than the incorrectly tagged products in the anti-His6 Western blots compared with the anti-FLAG Western blots.

The F. tularensis tmRNA has a GGC resume codon, rather than the canonical GCA resume codon found in most tmRNA ORFs from other bacterial species (25). Therefore, one intriguing possibility was that translation of the tmRNAFT hybrid in E. coli was not starting at the GGC resume codon but instead

FIGURE 4. Hybrid tmRNA variants result in robust but inaccurate tagging of a nonstop reporter. Western blot analysis of the λ-cl reporter tagging in the presence of SmpBWT-tmRNAH6 (A), the single hybrid (SmpBWT-tmRNAFT) (B), and double hybrid (SmpBFT-tmRNAFT) is shown. W3110 ΔsmpBssrA (DE3) strains with the λ-cl reporter and an SmpB-tmRNA plasmid were grown to OD600 of 0.5, and the cells were harvested after 3 h of reporter induction with IPTG. Lysates from an equal number of cells were resolved by electrophoresis and probed with anti-FLAG antibody. Shown are representative results from a set of three independent experiments, all of which were quantified and averaged to produce the ratio of tagged to untagged reporter (C) and the fraction of correctly tagged products from all three constructs (D). Error bars indicate S.D.; ****, p < 0.0005. His6 (H6).
was occurring at the GCA codon formed by the last two nucleotides of the resume codon and the first nucleotide of the second codon (GGC-AAC), producing a frame-shifted tagged product. It was also possible that translation was occurring in the correct frame but at a different start point. To distinguish between these possibilities, the tagged \( H_1 \) reporter proteins produced from a strain with hybrid SmpB FT and hybrid tmRNA FT-H6 were purified, resolved by electrophoresis on denaturing polyacrylamide gels, and the individual bands excised and subjected to mass spectrometry (LC-MS/MS). This analysis confirmed the assignment of the lowest molecular weight band as the untagged \( H_1 \) reporter (Fig. 9A). The identity of the highest molecular weight band was also verified as the reporter protein appended with the full-length tmRNA FT-H6 tag, in the correct reading frame, where the tag is added after the last reporter-encoded residue (Fig. 9B). Reporter proteins that migrated between the untagged form and the reporter appended with the correct full-length tag were also analyzed. This analysis showed that the masses of the additional tagged products closely matched the masses expected from reporter proteins appended with an incorrect tag that results from resumption of translation on tmRNA FT-H6 ORF in the \( H_1 \) reading frame (Fig. 9C). However, these data also matched a reporter appended with either a deamidated form of the full-length tag or a transcription terminator read-through product. We reason that the nature of the SmpB and tmRNA hybrids

FIGURE 5. Introduction of a His \(_6\) tag significantly weakens the tagging activity of the hybrid tmRNA. Representation of the sequence of the tmRNA ORF distal region in the hybrid encoding the natural \( F. tulariae \) tag (A) or the six-histidine (H6) substitution tag (B). C, Western blot analysis of the \( \lambda-cI \) reporter tagging in the presence of SmpB FT and tmRNA FT, either with the native \( F. tulariae \) tag or the His \(_6\) variant tag (tmRNA FT-H6). W3110 \( \Delta smpB \) BAR (DE3) strains with the \( \lambda-cI \) reporter plasmid and an SmpB-tmRNA plasmid were grown to a mid-log \( \text{OD}_{600} \), and the cells were harvested after 3 h of reporter induction with IPTG. Lysates from an equal number of cells were resolved by electrophoresis and probed with anti-FLAG antibody. The results shown are typical from a set of three independent experiments, all of which were quantified and averaged. The tagging activity (D) and accuracy (E) are represented in a similar fashion as the data in Fig. 4. Error bars indicate S.D.; **, \( p < 0.05 \); ***, \( p < 0.005 \). His \(_6\) (H6).
should not alter the level and ratio of the deamidated or tran-
scriptional read-through products. Therefore, we interpret
these data to be consistent with the reporter protein that results
from addition of a frame-shifted tag from tmRNAFT-H6, the
levels of which is expected to be influenced by alterations in key
residues of the SmpB protein and tmRNA.

Reversion of the Resume Codon Improves Activity in an
SmpB-dependent Manner—Our Western blot and LC-MS/MS
analyses suggested that the hybrid tmRNA constructs had
defects that resulted in improper selection of the resume codon,
irrespective of the identity of the SmpB C-terminal tail. This
defect was observed with both hybrid constructs encoding for
the tmRNAFT and tmRNAFT-H6. Therefore, it was likely that
the resume codon and its surrounding residues were responsi-
ble for the observed improper start codon selection with both
tmRNA hybrids. To directly examine this possibility, we
reverted the resume codon from the GGC codon found in
F. tularensis to the GCA codon found in E. coli. The GCA resume
codon reversion was introduced into the hybrid tmRNAFT-H6,
with either SmpBWT or the SmpBFT hybrid. In the context of
SmpBWT, the hybrid tmRNAFT-H6 construct with the GCA
resume codon displayed more accurate tagging than the GGC
codon control (Fig. 10). Despite this increase in accuracy, the
activity of both constructs was still weak relative to wild-type
tmRNA. The GCA hybrid tmRNAFT-H6, however, did not dis-
play improved tagging activity or accuracy during co-expres-
sion with the hybrid SmpBFT (Fig. 11, A–C). In fact, when co-
expressed with the hybrid SmpBFT, the GCA hybrid
tmRNAFT-H6 displayed lower activity relative to a GGC control
(Fig. 11B). To examine the possibility that this decrease in activ-
ity masked a change in tagging accuracy, we reverted the
resume codon in the tmRNAFT encoding the natural
F. tularensis tag; it should be noted that this construct displays
much more robust tagging activity compared with hybrid
tmRNAH6. When expressed in the context of the SmpBFT, the
GCA tmRNAFT-H6 hybrid was both as robust and inaccurate as
the corresponding GGC control. Collectively, these data sug-
gest that in cooperation with the SmpB C-terminal tail, ele-
ments within tmRNA and the ribosome act to recognize and set
the GCA codon as the resume point for trans-
translation.

Contributions of the Resume Codon Proximal Portion of
the tmRNA ORF and the SmpB C-terminal Tail to Reading Frame
Establishment—The mRNA-like region of tmRNA is rich in
information content, and the encoded peptide tag sequence
contains signals for one or more cellular proteases (7, 8, 16, 19).
Several conserved nucleotides are essential constituents of this
region, alterations of which have severe consequences on
tmRNA tagging activity. For instance, substitution of the ade-
mine at position 86 (A86) with cytosine in the E. coli tmRNA
nearly abolishes all tagging activity (30, 40). This particular sub-

FIGURE 6. Hybrid SmpB rescues the activity defect of the hybrid tmRNAFT-H6. A, Western blot analysis of the λ-ct reporter tagging in the presence of either the SmpBWT-tmRNAH6, the hybrid tmRNAFT-H6 paired with SmpBWT, or the hybrid tmRNAFT-H6 hybrid paired with SmpBFT. The λ-ct nonstop reporter (λ-ct), which elicits ribosome stalling, was used as the test reporter, and λ-ct-stop (λ-ct), which does not elicit ribosome stalling and promotes efficient translation termination, was used as a control reporter. W3110 ΔsmpBΔsra (DE3) strains with the respective λ reporter plasmid and specified SmpB-tmRNA variant were grown to a mid-log OD600 n and the cells were harvested after 3 h of reporter induction with IPTG. Lysates from an equal number of cells were resolved by electrophoresis and probed with anti-FLAG antibody. The results shown are typical from a set of three independent experiments, all of which were quantified and averaged. The tagging activity (B) and accuracy
(C) are represented in a similar fashion as the data in Fig. 4. Error bars indicate S.D.; *** p < 0.005; **** p < 0.0005. His6 (H6).

SmpB C Terminus and Hinge Are Required for Ribosome Rescue
stitution is thought to affect tmRNA function by stabilizing an inhibitory hairpin, through interactions between cytosine 86 and the naturally occurring guanine 99, forming a C86:G99 base pair (Fig. 11D). This rationalization appears insufficient to explain the tagging defect because analysis of a variant with the reciprocal pair of residues, a G86:C99 base pair, is still functional (Fig. 11E). Furthermore, the defect associated with the 86A→C substitution can be rescued by additional compensatory mutations in the region (Fig. 11E and F). These observations indicate a complex set of determinants for trans-translation function in this region.

**DISCUSSION**

Contribution of the C-terminal Tail and A-site Proximal Residues to SmpB Function—Previous studies have reported biochemical, structural, and genetic evidence of a functional interaction between the SmpB C-terminal tail and the mRNA-like domain of tmRNA (27, 30–32). The SmpB C-terminal tail is required for a step after ribosome binding but prior to transpeptidation (27). It was thus plausible that it also played a key role in the establishment and selection of the correct tmRNA reading frame. Our data suggest that not only is the C-terminal tail itself important but residues in the SmpB hinge region, near the junction of the protein body and the tail, also contribute to a novel SmpB function in trans-translation. This unique region includes a highly conserved glycine at position 132 and the neighboring lysine residues, Lys-131, Lys-133, and Lys-134. Our mutational analysis of the highly conserved Gly-132 supports the hypothesis that this residue imparts flexibility to the C-terminal tail of SmpB to enable crucial contacts before and after accommodation of the tmRNA ORF as a surrogate mRNA template. Consistent with this hypothesis, we have found that substitution of Gly-132 with a bulky or charged residue significantly decreases tagging activity, although small or uncharged amino acids appear to be tolerated at this position. These findings suggest that there are precise steric requirements for the amino acid at position 132, which can be optimally fulfilled by glycine. We found that alanine substitution at this position, or substitution of conserved lysines in the region encompassing residues 131–134, decreased the tagging ability of SmpB. This finding is qualitatively consistent with previous studies (30), although the defects we observed were not as severe. Nonetheless, the SmpBG132D and SmpBK131A/K133A/K134A substitutions demonstrate that this region plays a critical role in SmpB function during trans-translation. Our studies also suggest that the spatially proximal N-terminal residues, Asn-17, Lys-18, and Arg-19, do not appear to play a vital role in tmRNA tagging.
activity. Indeed, the entire region up to residue 21 appears to be dispensable. This is surprising due to the high degree of conservation of these residues and their spatial proximity to decoding center and the conserved 131–134 region of SmpB. We cannot exclude the possibility that these residues are functionally redundant with another part of the protein. It is also conceivable that these residues function in an aspect of trans-translation that we have not yet examined.

In an effort to further characterize the role of resume codon flanking residues in trans-translation activity of tmRNA, as well as potential interactions between this region and the SmpB C-terminal tail, we created hybrid variants of both molecules. We observed that replacement of the mRNA-like domain of tmRNA with the equivalent sequence from \( F. \) tularensis resulted in tagging of the \( /H9261 \)-cI reporter and production of multiple tagged species. Mass spectrometry analysis of these products confirmed the presence of both the untagged reporter and the reporter appended with the full-length tag. The LC-MS/MS data also revealed the identity of the two additional bands as products of +1 and –1 frameshift on hybrid tmRNA ORF. We interpret the results to be consistent with the conclusion that the substitution of the tmRNA ORF with the equivalent sequence from the \( F. \) tularensis tmRNA leads to a defect in resume codon selection without negatively impacting reading frame engagement.

Translation of the tag sequence in the hybrid tmRNA\(^{FT}\), while resuming in an inconsistent fashion, was robust both in the presence of SmpB\(^{WT}\) and SmpB\(^{FT}\) hybrid. Slight decreases in tagging activity, as well as a defect in nonstop mRNA degradation, have been observed when the \( E. \) coli tmRNA\(^{H6}\) variants were analyzed (23). The severity of the defect when the His\(_6\) motif was introduced into the tmRNA\(^{FT}\) hybrid was unexpected. This defect in activity was rescued by expression of the cognate SmpB\(^{FT}\) hybrid. Thus, the influence exerted by tmRNA residues in the resume codon proximal region appears to be
dependent on a sequence-specific interaction with the C-terminal tail of SmpB. Furthermore, our data suggest that these residues influence engagement of the reading frame in a manner distinct from their impact on resume codon selection. Interestingly, neither introduction of the hybrid sequence nor sequence encoding an His$_{6}$ tag alone is sufficient for the large decrease in activity.

**Contribution of the Distal Portion of the tmRNA ORF and the SmpB C-terminal Tail to Reading Frame Establishment**—The distal region of the mRNA-like domain of tmRNA plays a variety of roles in trans-translation. This region encodes for the last few amino acids of the tag and contributes to the formation of the structurally important helix 5, the integrity of which is important for tagging activity. Our data suggest that this distal region also plays a sequence-specific role in the engagement of the tmRNA ORF by the ribosome. Changes to residues within the last two codons of the tmRNA ORF appear to contribute the most to the phenotype observed in the tmRNA$^{FT-146}$ hybrid. This result is not surprising, as these residues overlap with determinants critical for degradation of nonstop mRNAs (23). Because the ribosome adopts a closed conformation around the mRNA template during translation, structural rearrangements are likely to be required to enable engagement of the tmRNA ORF. We propose that residues in the last two codons of the tmRNA ORF play an important role in the transition of the ribosomal mRNA channel to an open conformation. Our findings suggest that the entry of tmRNA into the ribosomal mRNA channel depends on multiple supplementary determinants, which include the C-terminal tail of SmpB and both the proximal and the distal portions of the mRNA-like domain of tmRNA. In this model, these determinants form interactions between SmpB and tmRNA, as well as the ribosome, to both engage the tmRNA reading frame and select the appropriate resume codon.

**FIGURE 9. LC-MS/MS analysis of the untagged and tagged versions of $\lambda$-cl reporter.** A, amino acid sequence of the C termini of the $\lambda$-cl reporter protein peptides (with zero, one, or two phenylalanine residues) is shown in black. Identity of this peptide was verified through MS/MS fragment ion analysis. The y-ions are shown in blue, and the b-ions are shown in green. B, junction peptide and the associated spectra of the $\lambda$-cl reporter protein appended with the full-length tmRNA$^{FT-146}$ tag. The reporter protein sequence is shown in black, and amino acids provided by the tmRNA$^{FT-146}$ tag are shown in pink. Arrows indicate the location of ions on the spectra. The inset shows a portion of the MS spectra of the parental ions. C, MS/MS fragmentation spectra that closely match the junction peptide of the reporter appended with the $\pm 1$ frame-shifted tmRNA$^{FT-146}$ tag. Important sections of these spectra, showing the fragmentation pattern of the frame-shifted tag, are highlighted. The reporter protein sequence is shown in black and the appended tag sequence in pink.
Interestingly, potential interactions between the distal portion of the mRNA-like domain of tmRNA and ribosomal proteins S2 and S3 were observed in a recent structural study of a tmRNA translocation intermediate (36). In particular, residues 119–122 in *E. coli* tmRNA were predicted to interact with ribosomal protein S2. This prediction is consistent with our findings, because the region in the hybrid tmRNA partially overlaps with the last two codons. Likewise, residues 129 and 130, which are thought to interact with ribosomal protein S3 (36), also correspond to residues altered in the two-His substitution of the ultimate and penultimate codons. Ribosomal proteins S2 and S3 help form a latch at the solvent side of the mRNA channel. Opening this latch might be required for permitting tmRNA access to the ribosomal mRNA channel. Our findings are thus consistent with the proposed model that this set of tmRNA-S2/S3 interactions are important for stabilization of a wide open conformation of the ribosomal mRNA entry channel, which in turn is necessary for the accommodation of the mRNA-like domain of tmRNA.

### Resume Codon Proximal Region and the SmpB C-terminal Tail Influence Reading Frame Selection

To establish the tmRNA ORF as the surrogate template, two events must occur. First, the mRNA-like domain must gain access to the ribosomal mRNA channel. Following or concurrently with this, the ribosome must select the correct reading frame to resume translation. Our studies of the hybrid tmRNA show that the ribosome selects the resume codon in an inaccurate manner. We propose that this is a reflection of an innate preference of the *E. coli* ribosome for the GCA resume codon. Indeed, reversion to this resume codon, from GGC to GCA, improved the accuracy with which hybrid tmRNA*FT-H6* was able to append the tag. This effect, however, was dependent on the presence of the cognate SmpB C-terminal tail, which has co-evolved a preference for the respective resume codon. From these data, we conclude that resume codon selection is a distinct process from engagement of the reading frame and utilizes a separate set of determinants. These data also suggest that interactions important for this activity lie within the SmpB C-terminal tail, the tmRNA resume codon and its flanking residues, and the ribosome. Accurate resume codon selection thus occurs only when all interactions are properly and synchronously established.

In light of these findings, and consistent with all previous data, we propose a model for the role of conserved regions of the SmpB protein and tmRNA in accurate selection of the resume codon during trans-translation. We have previously demonstrated that the C-terminal tail of SmpB plays a crucial role in a step after recognition and binding of the SmpB-tmRNA complex to stalled ribosomes (13, 27). Recent structural studies show that the C-terminal tail of SmpB resides in the mRNA channel of the rescued ribosome (28), and in the subsequent step, the ribosomal mRNA channel assumes a wide open conformation to accommodate entry of the mRNA-like domain of tmRNA (36). We propose that the SmpB C-terminal tail makes critical contacts with ribosomal elements to promote opening of the ribosomal mRNA channel and that residues in

![FIGURE 10. Reversion of the resume codon to GCA improves tagging accuracy.](image-url)
the stop codon proximal region of the tmRNA ORF, namely the ultimate and penultimate codons, make supplementary contacts that facilitates this opening. Our data also suggest that the C-terminal tail of SmpB must make crucial contacts with the tmRNA ORF to facilitate the ensuing selection of the correct resume codon. However, the SmpB C-terminal tail must subsequently vacate the mRNA entry channel for resumption of translation on the tmRNA ORF. We propose that the SmpB Gly-132 region acts as a hinge to permit the C-terminal tail to move out of the ribosomal mRNA channel. This unique SmpB hinge region must have sufficient flexibility to enable the various functions of its C-terminal tail, and the sterically unrestricted glycine is ideally suited for this function. Other small amino acids that permit sufficient flexibility can be substituted at this position but not without some loss of function. Clearly, large polar residues severely diminish the required flexibility, causing potential steric clashes with the mRNA channel and the tmRNA ORF, and are thus strongly disfavored at this position. Presented here are thus several novel and distinct determinants in SmpB protein and tmRNA that play a crucial role in overall activity and accurate establishment of the tmRNA reading frame during trans-translation.

FIGURE 11. Reversion of the resume codon to GCA does not improve tagging accuracy in the presence of hybrid SmpB*. A, Western blot analysis of reporter tagging in the presence of the hybrid SmpB* with hybrid tmRNA* containing either the GGC or GCA resume codon. W3110 ΔsmpBssrA (DE3) strains with the λ-cl reporter plasmid and the indicated SmpB-tmRNA plasmid were grown to a mid-log OD600 and cells were harvested after 3 h of reporter induction by IPTG. Lysates from an equal number of cells were resolved by electrophoresis and probed with anti-FLAG antibody. The tagging activity (B) and accuracy (C) are represented in a similar fashion as the data in Fig. 4. D, schematic representation of proposed hairpin structure formed by the A86C mutation. Positions of residues Cys-86, Gly-99, and A93Δ frameshift mutations are indicated. Different substitutions at position 86 and 99 were introduced to test the effect of hairpin formation. These variants were then assayed for their ability to support the growth of the λimmP22 c2-5dis hybrid phage. E, quantitative results of three independent experiments, normalized for wild-type sequence (Ala-86 and Gly-99), are shown. F, mutations were introduced to shift the reading frame of the tmRNA ORF. These mutations compensated for the phage growth defect of 86A-99C. Error bars indicate S.D.; data are plotted on a log scale and normalized to wild-type sequence in each case. The results shown are typical from a set of three independent experiments, all of which were quantified and averaged. Error bars indicate S.D.; ****, *p < 0.0005. His6 (H6).
REFERENCES

1. Laursen, B. S., Sørensen, H. P., Mortensen, K. K., and Sperling-Petersen, H. U. (2005) Initiation of protein synthesis in bacteria. Microbiol. Mol. Biol. Rev. 69, 101–123

2. Zheng, X., Hu, G.-Q., Sha, Z.-S., and Zhu, H. (2011) Leaderless genes in bacteria: clues to the evolution of translation initiation mechanisms in prokaryotes. BMC Genomics 12, 361

3. Wills, N. M., O’Connor, M., Nelson, C. C., Retberg, C. C., Huang, W. M., Gesteland, R. F., and Atkins, J. F. (2008) Translational bypassing without peptidyl-tRNA anticondor scanning of coding gap mRNA. EMBO J. 27, 2533–2544

4. Hayes, C. S., and Keiler, K. C. (2010) Biology of trans-translation. Annu. Rev. Microbiol. 62, 133–151

5. Moore, S. D., and Sauer, R. T. (2007) The tmRNA system for translational surveillance and ribosome rescue. Annu. Rev. Biochem. 76, 101–124

6. Dulebohn, D., Choy, I., Sundermeier, T., Okan, N., and Karzai, A. W. (2007) Trans-translation: the tmRNA-mediated surveillance mechanism for ribosome rescue, directed protein degradation, and nonstop mRNA decay. Biochemistry 46, 4681–4693

7. Karzai, A. W., Roche, E. D., and Sauer, R. T. (2000) The SsrA-TmRNA system for protein tagging, directed degradation and ribosome rescue. Nat. Struct. Biol. 7, 449–455

8. Barends, S., Kraal, B., and van Wexel, G. P. (2011) The tmRNA-tagging mechanism and the control of gene expression: a review. Wiley Interdiscip. Rev. RNA 2, 233–246

9. Janssen, B. D., and Hayes, C. S. (2012) The tmRNA ribosome-rescue system. Adv. Protein Chem. Struct. Biol. 86, 151–191

10. Withey, J. H., and Friedman, D. I. (2003) A salvage pathway for protein structures: tmRNA and trans-translation. Annu. Rev. Biochem. 57, 101–123

11. Withey, J. H., and Friedman, D. I. (2002) The biological roles of trans-translation. Curr. Opin. Microbiol. 5, 154–159

12. Sundermeier, T. R., and Karzai, A. W. (2007) Functional SmPB-ribosome interactions require tmRNA. J. Biol. Chem. 282, 34779–34786

13. Barends, S., Karzai, A. W., Sauer, R. T., Wower, J., and Kraal, B. (2001) Simultaneous and functional binding of SmPB and EF-Tu-TF to the alanyl acceptor arm of tmRNA. J. Mol. Biol. 314, 9–21

14. Karzai, A. W., Susskind, M. M., and Sauer, R. T. (1999) SmPB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). EMBO J. 18, 3793–3799

15. Baker, T. A., and Sauer, R. T. (2006) ATP-dependent proteases of bacteria: recognition logic and operating principles. Trends Biochem. Sci. 31, 647–653

16. Baker, T. A., and Sauer, R. T. (2012) ClpXP, an ATP-powered unfolding and protein-degradation machine. Biochim. Biophys. Acta 1823, 15–28

17. Choy, I. S., Aung, L. L., and Karzai, A. W. (2007) Lon protease degrades transfer-messenger RNA-tagged proteins. J. Bacteriol. 189, 6564–6571

18. Ge, Z., and Karzai, A. W. (2009) Co-evolution of multipartite interactions between an extended tmRNA tag and a robust Lon protease in Mycoplasma. Mol. Microbiol. 74, 1083–1099

19. Sauer, R. T., and Baker, T. A. (2011) AAA+ proteases: ATP-fueled machines of protein destruction. Annu. Rev. Biochem. 80, 587–612

20. Ge, Z., Mehta, P., Richards, J., and Karzai, A. W. (2010) Non-stop mRNA decay initiates at the ribosome. Mol. Microbiol. 78, 1159–1170

21. Richards, J., Mehta, P., and Karzai, A. W. (2006) RNase R degrades non-stop mRNAs selectively in an SmPB-tRNA-dependent manner. Mol. Microbiol. 62, 1700–1712

22. Mehta, P., Richards, J., and Karzai, A. W. (2006) tmRNA determinants required for facilitating nonstop mRNA decay. RNA 12, 2187–2198

23. Okan, N. A., Bliska, J. B., and Karzai, A. W. (2006) A role for the SmPB-SsrA system in Yersinia pseudotuberculosis pathogenesis. PLoS Pathogens 2, e6

24. Svetlanov, A., Puri, N., Mena, P., Koller, A., and Karzai, A. W. (2012) Francisella tularensis tmRNA system mutants are vulnerable to stress, avirulent in mice, and provide effective immune protection. Mol. Microbiol. 85, 122–141

25. Dulebohn, D. P., Cho, H. J., and Karzai, A. W. (2006) Role of conserved surface amino acids in binding of SmPB to SsrA RNA. J. Biol. Chem. 281, 28536–28545

26. Sundermeier, T. R., Dulebohn, D. P., Cho, H. J., and Karzai, A. W. (2005) A previously uncharacterized role for small protein B (SmPB) in transfer messenger RNA-mediated trans-translation. Proc. Natl. Acad. Sci. U.S.A. 102, 2316–2321

27. Neubauer, C., Gillet, R., Kelley, A. C., and Ramakrishnan, V. (2012) Decoding in the absence of a codon by tmRNA and SmPB in the ribosome. Science 335, 1366–1369

28. Cheng, K., Ivanova, N., Scheres, S. H., Pavlov, M. Y., Carazo, J. M., Hebert, H., Ehrenberg, M., and Lindahl, M. (2010) tmRNA: SmPB complex mimics native aminocyl-tRNAs in the A site of stalled ribosomes. J. Struct. Biol. 169, 342–348

29. Miller, M. R., Liu, Z., Cazier, D. I., Gebhard, G. M., Herron, S. R., Zaher, H. S., Green, R., and Buskirk, A. R. (2011) The role of SmPB and the ribosomal decoding center in licensing tmRNA entry into stalled ribosomes. RNA 17, 1727–1736

30. Watts, T., Cazier, D., Healey, D., and Buskirk, A. (2009) SmPB contributes to reading frame selection in the translation of transfer-messenger RNA. J. Mol. Biol. 391, 275–281

31. Konno, T., Kurita, D., Takada, K., Muto, A., and Himeono, H. (2007) A functional interaction of SmPB with tmRNA for determination of the resuming point of trans-translation. RNA 13, 1723–1731

32. Nonin-Lecomte, S., Germain-Amiot, N., Gillet, R., Hallier, M., Ponchon, L., Dardel, F., and Felden, B. (2009) Ribosome hijacking: a role for small protein B during trans-translation. EMBO Rep. 10, 160–165

33. Metzlinger, L., Hallier, M., and Felden, B. (2008) The highest affinity binding site of small protein B on transfer messenger RNA is outside the tRNA domain. RNA 14, 1761–1772

34. Yusupova, G. Z., Yusupov, M. M., Cate, J. H., and Noller, H. F. (2001) The path of messenger RNA through the ribosome. Cell 106, 233–241

35. Tarmath, D. J., Yamamoto, H., Rother, K., Wittek, D., Pech, M., Mielke, T., Loerke, J., Scheerer, P., Ivanov, P., Teraoka, Y., Shpanchenko, O., Nierhaus, K. H., and Spahn, C. M. (2012) The complex of tmRNA-SmPB and EF-G on translocating ribosomes. Nature 485, 526–529

36. Sundermeier, T., Ge, Z., Richards, J., Dulebohn, D., and Karzai, A. W. (2008) Studying tmRNA-mediated surveillance and nonstop mRNA decay. Methods Enzymol. 447, 329–358

37. Withey, J., and Friedman, D. (1999) Analysis of the role of trans-translation in the requirement of tmRNA for AimP22 growth in Escherichia coli. J. Bacteriol. 181, 2148–2157

38. Okan, N. A., Mena, P., Benach, J. L., Bliska, J. B., and Karzai, A. W. (2010) The SmPB-SsrA mutant of Yersinia pestis functions as a live attenuated vaccine to protect mice against pulmonary plague infection. Infect. Immun. 78, 1284–1293

39. Williams, K. P., Martindale, K. A., and Bartel, D. P. (1999) Resuming translation on tmRNA: a unique mode of determining a reading frame. EMBO J. 18, 5423–5433

40. Bensho, Y., Shibata, R., Sekine, S., Murayama, K., Higashijima, K., Hori-Takemoto, C., Shirouzu, M., Kuramitsu, S., and Yokoyama, S. (2007) Structural basis for functional mimicry of long-variable-arm tRNA by transfer-messenger RNA. Proc. Natl. Acad. Sci. U.S.A. 104, 8293–8298