Bacteria possess a unique salvage mechanism for rescuing ribosomes stalled on aberrant mRNAs. A complex of SmpB protein and SsrA RNA orchestrates this salvage process. The specific and direct binding of SmpB facilitates recognition and delivery of SsrA RNA to stalled ribosomes. The SmpB protein is conserved throughout the bacterial kingdom and contains several conserved amino acid sequence motifs. We present evidence to demonstrate that amino acid residues Glu-31, Leu-91, and Lys-124, which are highly conserved and clustered along an exposed surface of the protein, play a crucial role in the SsrA-mediated peptide tagging process. Our analysis suggests that the peptide-tagging defect exhibited by these SmpB variants is due to their inability to facilitate the delivery of SsrA RNA to stalled ribosomes. Moreover, we present evidence to demonstrate that the ribosome association defect of these variants is due to their reduced SsrA binding affinity. Consistent with these findings, we present biochemical evidence to demonstrate that residues Glu-31, Leu-91, and Lys-124 are essential for the SsrA binding activity of SmpB protein. Furthermore, we have investigated the interactions of SmpB-SsrA orthologues from the thermophilic bacterium *Thermoanaerobacter tengcongensis*. Our investigations demonstrate an analogous role for the equivalent T. tengcongensis residues in SmpB-SsrA interactions, hence further validating our findings for the *Escherichia coli* SmpB-SsrA system. These results demonstrate the functional significance of this cluster of conserved residues in SmpB binding to SsrA RNA, suggesting they might represent a core contact surface for recognition of SsrA RNA.

Specific complexes of RNA and protein perform many essential biological functions, including RNA processing, RNA turnover, RNA transport, and RNA folding as well as the translation of genetic information from mRNA into protein sequences. Principles that govern RNA-protein interactions are inadequately understood due in large part to a paucity of detailed structural and biochemical information on RNA-protein complexes. These principles are important for understanding RNA-protein machines, such as the ribosome and RNA-protein structure and function in general.

SsrA (also known as transfer mRNA and 10Sa RNA) is a small, highly structured RNA that is found in all bacteria (1–3). SsrA, through its unique sequence and structure, is endowed with both tRNA and mRNA-like functions (1, 3–9). The tmRNA model for SsrA function proposes that alanine-charged SsrA RNA recognizes stalled ribosomes, binds at the ribosomal A-site, and donates its alanine charge to the growing polypeptide (11–13). The ribosomal reading frame then switches to the mRNA segment of SsrA, adding a degradation tag to the C terminus of targeted polypeptide. SsrA-tagged proteins are then recognized by cellular proteases and efficiently degraded (1, 3, 7–9). This unique bacterial process is also known as trans-translation. All known biological activities of SsrA RNA require small protein B (SmpB) (1, 14). The known functions of SmpB are specific binding to SsrA RNA and promoting stable association and proper engagement of the SmpB-SsrA complex with stalled ribosomes (1, 14, 15). In the absence of SmpB protein SsrA does not associate stably with 70 S ribosomes. SmpB binds specifically to the tRNA-like domain of SsrA and, although not required, enhances the efficiency of its aminoacylation by stabilizing SsrA tertiary structure (16). Therefore, formation of the SmpB-SsrA complex appears to be critical for recognition and rescue of stalled ribosomes.

The SmpB protein is highly conserved in eubacteria. Sequence alignment analysis of SmpB orthologues from 115 bacterial species shows the presence of several conserved polar and hydrophobic residues. In studies described herein, we have sought to gain a better understanding of the biological role and energetic contributions of highly conserved surface amino acids of SmpB protein. We have specifically focused on a number of conserved residues that are closely clustered on one surface of the SmpB protein. We provide in vivo and in vitro evidence for the contribution of these amino acids in trans-translation. Additionally, we have investigated the SmpB-SsrA RNA complex of the thermophilic bacterium *Thermoanaerobacter tengcongensis* (Tten)2 and demonstrate that the equivalent residues make significant energetic contributions to the binding of Tten SmpB protein to Tten SsrA RNA. These results are consistent with the conclusion that this cluster of surface residues represents the core contact points of SmpB-SsrA interactions that is conserved across divergent bacterial species.

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2 The abbreviations used are: Tten, *T. tengcongensis*; N-Tricine, [2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; WT, wild type.
MATERIALS AND METHODS

Site-directed Mutagenesis—All single and double SmpB mutations were introduced by PCR mutagenesis using the Stratagene QuikChange kit. Plasmid pET28 BA\(^{His6}\) (15) codes for SsrA\(^{His6}\). SsrA RNA with a modified mRNA-like domain coding for a six-histidine tag.

Ribosome Association Assays—For 70 S ribosome preparations, 750-ml cultures of W3110 ΔsmpB/(DE3) containing plasmid pET28 BA\(^{WT}\) or pET28BA with specified SmpB amino acid substitutions were grown in LB containing 3 μM isopropyl 1-thio-β-D-galactopyranoside to \(A_{600}\) of 0.8–1.0. Bacterial cells were harvested, washed in 50 mM Tris (pH 7.5), pelleted, and stored at −80 °C. Cell pellets were resuspended in buffer A (20 mM Tris (pH 7.5), 300 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 gentle sonication. Lysates were centrifuged at 33,000 \(\times\) g for 30 min. Supernatants were transferred to new tubes and centrifuged again at 33,000 \(\times\) g for 30 min. Typically, a 19-ml aliquot of the supernatant was layered onto a 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 (Ambion)) and centrifuged at 85,000 \(\times\) g for 22 h. The pellet containing tight-coupled ribosomes was washed twice with 5 ml of cold buffer B. Pellets were resuspended in 0.25 ml of buffer A, and equivalent numbers of ribosomes were loaded onto a 10–50% sucrose cushion in buffer A. The sucrose gradients were centrifuged at 42,000 \(\times\) g for 17 h. Gradients were fractionated, and the fractions corresponding to purified 70 S ribosomes were pooled and used for Western and Northern blot analysis. 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 (Ambersham Biosciences), and probed with a psoralen-modified psoralen-biotin (Ambion) labeled full-length SsrA probe. For Western blot analysis, an equal number of ribosomes were loaded per lane, and the associated proteins were resolved on 15% Triscine gels. Western blots were developed with antibodies raised against purified SmpB protein and a secondary IR 800-nm dye conjugated secondary antibody (Molecular Probes).

Purification of SmpB Alanine-substituted Variants—Bacterial strain BL21 (DE3)/pEysS (Stratagene) was transformed with plasmid pET28 BA\(^{His6}\) harboring SmpB\(^{WT}\) or one of the alanine variants: SmpB\(^{E31A}\), SmpB\(^{K124A}\), SmpB\(^{Q94A}\), SmpB\(^{E31A/L91A}\), SmpB\(^{E31A/L124A}\), SmpB\(^{E93A/Q94A}\), SmpB\(^{E31A/L91A/K124A}\), and SmpB\(^{E31A/L91A/Q94A}\). Endogenous protein tagging assays were performed as previously described (15).

Western and Northern blot analysis. RNA for Northern blot analysis was extracted with Tri-LS reagent (Molecular Research), and probed with a psoralen-modified psoralen-biotin (Ambion) labeled full-length SsrA probe. For Western blot analysis, an equal number of ribosomes were loaded per lane, and the associated proteins were resolved on 15% Triscine gels. Western blots were developed with antibodies raised against purified SmpB protein and a secondary IR 800-nm dye conjugated secondary antibody (Molecular Probes).

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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 (3 × 30-s pulses, with the addition of 0.1 ml of 0.1 M phenylmethylsulfonyl fluoride after each pulse). Cell lysates were centrifuged for 1 h at 15,000 rpm in an SS-34 rotor to remove cellular debris. Due to the greater thermal stability of the Tten SmpB protein, an additional heat treatment step was included for wild-type Tten SmpB protein and all of its alanine substitution variants. The S30 supernatants of Tten SmpB proteins were heated at 65 °C for 10 min. This treatment results in the denaturation and precipitation of greater than 80% of soluble E. coli proteins, whereas the Tten SmpB variants are entirely unaffected and remain soluble. The heat-treated protein samples were centrifuged for 1 h at 15,000 rpm to pellet the denatured E. coli proteins. The supernatant fractions were mixed with 2 ml of Ni\(^{2+}\)–NTA resin (Qiagen) and pre-equilibrated in lysis buffer, and the binding reaction was permitted to proceed with gentle rocking for 1 h at 4 °C. The nickel–nitrilotriacetic acid resin was applied to a chromatography column and washed 3 × with 50 ml of lysis buffer. Proteins were eluted with 12 ml of elution buffer (150 mM KCl, 50 mM Tris (pH 8.0), 200 mM imidazole, 20 mM β-mercaptoethanol). The eluates were then diluted 3-fold in fast protein liquid chromatography buffer A (50 mM KCl, 50 mM Hepes (pH 7.5), 5 mM MgCl\(_2\), 2 mM β-mercaptoethanol) and applied onto a Mono S ion exchange column (Amersham Biosciences–GE Healthcare). A gradient of 50–850 mM KCl in buffer A was developed over 20 column volumes to isolate the SmpB protein. SmpB protein, with greater than 95% purity, elutes at around 500 mM KCl under these conditions. Protein concentrations were determined by absorbance at 280 nm using extension coefficients of 29575 M\(^{-1}\) cm\(^{-1}\) for the E. coli SmpB variants and 10430 M\(^{-1}\) cm\(^{-1}\) for the Tten SmpB variants. Protein aliquots were stored at −80 °C until needed.

Electrophoretic Mobility-shift Assays—SsrA variants were produced and labeled as described previously (15). The csrB gene was PCR-amplified from E. coli genomic DNA using a 5-primer csrb2 (‘5’-CGAATTCTAATAGCCTACTATAGGTGTTCCTCAGGCAAGAAC-3’) and a 3-primer csrb3 (‘5’-AAAGGAGGTACTGTTTTACCAG-3’). The amplified product was purified by gel electrophoresis and re-amplified to incorporate a T7 promoter sequence at its 5-end. CsrB RNA was transcribed using T7 RNA polymerase in accord with the manufacturer’s recommendations (U. S. Biochemical Corp.). Template DNA was digested with DNase I, and the transcription products were phenol/chloroform-extracted and purified by electrophoresis on denaturing polyacrylamide gels. Electrophoretic mobility-shift assays were performed essentially as described (15), with minor modifications. Briefly, E. coli and T. tengcongensis SmpB protein variants were diluted to the desired protein concentrations in electrophoretic mobility-shift buffer (50 mM Tris (pH 7.5), 2 mM MgCl\(_2\), 300 mM KCl, 100 μg/ml bovine serum albumin, 2 mM β-mercaptoethanol 0.01% Nonidet P-40 (v/v), 10% glycerol (v/v)) in the presence of 100 nM CsrB RNA as a nonspecific competitor (in 100-fold excess over the specific SsrA RNA). Approximately 100 fmol of 3-end-labeled SsrA\(^{143}\) RNA (~1000 cpm/reaction) were
added to each tube and incubated at room temperature for 30 min. Samples were loaded on a 12% non-denaturing gel and resolved by electrophoresis at 200 V in 0.5\%/H11003 Tris borate-EDTA to resolve the free RNA from RNA-protein complexes. Gels were run at 4 °C, dried, and exposed overnight to phosphorimaging screens. All binding experiments for \textit{E. coli} and \textit{T. tengcongensis} SmpB variants were performed at least in triplicate, covering a protein concentration range of 0.1 nM to 1.5 \%/H9262 M.

Data analysis was performed according to Berggrun and Sauer (17). Briefly, the fraction of the primary bound species at each SmpB concentration was determined, and the apparent equilibrium dissociation constant was obtained by curve-fitting using the equation $\Theta_{eq} = C/(1 + K_d/[\text{SmpB}])$, where $\Theta_{eq}$ is the fraction of RNA bound at equilibrium, $C$ is a constant representing the maximum fraction bound of the specific bound species, and $[\text{SmpB}]$ is the initial concentration of SmpB.

RESULTS

Mutations in Conserved SmpB Amino Acids Decrease the Level of Tagged Proteins in Vivo—The SmpB protein is a requisite component of the \textit{trans}-translation process. It performs several key functions, including specific binding to SsrA RNA, recognition of stalled ribosomes, and proper positioning of SsrA in the ribosomal A-site (1, 3, 14, 15). Sequence alignment analysis of SmpB protein revealed the identity of a number of invariant amino acid residues that are conserved across many diverse bacterial species (Fig. 1A). To gain insights into the functional significance of these highly conserved amino acids, we carried out a systematic alanine-scan mutagenesis of strategic residues of the \textit{E. coli} SmpB protein. This analysis enabled us to evaluate the contribution of conserved residues to known SmpB functions in \textit{trans}-translation. We previously reported on the functional significance of a number of highly conserved amino acid residues located in the C-terminal tail of the SmpB protein (15). In this study we focused specifically on several evolutionarily conserved amino acid residues located in the C-terminal tail of the SmpB protein (15). In this study we focused specifically on several evolutionarily conserved amino acid residues located in the C-terminal tail of the SmpB protein (15). In this study we focused specifically on several evolutionarily conserved amino acid residues located in the C-terminal tail of the SmpB protein (15). In this study we focused specifically on several evolutionarily conserved amino acid residues located in the C-terminal tail of the SmpB protein (15). In this study we focused specifically on several evolutionarily conserved amino acid residues located in the C-terminal tail of the SmpB protein (15).
using pET28BA\textsuperscript{His\textsubscript{6}}, a plasmid that harbors the smpB gene and a functional ssrA variant (ssrA\textsuperscript{His\textsubscript{6}}) that encodes ANDEHHH-HHH in place of the normal ANDENYALAA degradation tag. The SsrA\textsuperscript{His\textsubscript{6}} variant permits endogenously tagged proteins to be purified by nickel-nitrilotriacetic acid affinity chromatography and detected by Western blot analysis.

To scrutinize the contributions of individual residues to known SmpB functions, we evaluated the effect of single alanine substitutions on the SmpB-SsrA mediated trans-translation process in vivo. To this end, we transformed an smpB deletion strain with the pET28BA\textsuperscript{His\textsubscript{6}} plasmid, harboring a single alanine substitution mutant of SmpB. We purified endogenously His\textsubscript{6} tagged products of the SmpB-SsrA system and resolved them by electrophoresis on SDS-polyacrylamide gels. The levels of endogenously tagged proteins were determined by Western blot analysis using anti-His\textsubscript{6} antibodies. This analysis revealed that alanine substitutions of the two variably conserved SmpB residues (Asn-93 and Gln-94) did not have a substantial effect on the ability of SmpB to support tagging of endogenous substrates (data not shown). In contrast, alanine substitutions of SmpB residues Glu-31, Leu-91, and Lys-124 resulted in a modest and reproducible decrease in the level of endogenously tagged proteins. Compared with wild-type SmpB protein, these variants consistently displayed a 15–20% reduction in endogenous tagging activity, suggesting a role for these highly conserved residues in one of the known SmpB functions (data not shown).

To gain further insight into the contributions of these residues, we generated a number of double alanine substitution variants of SmpB protein, including E31A/L91A (SmpB\textsuperscript{EL}), E31A/K124A (SmpB\textsuperscript{EK}), L91A/K124A (SmpB\textsuperscript{LK}), and N93A/Q94A (SmpB\textsuperscript{NQ}). Evaluation of the ability of the double alanine substitution variant SmpB\textsuperscript{NQ} to support trans-translation in vivo revealed little or no loss of tagging activity. In contrast, double-alanine-substituted variants SmpB\textsuperscript{EL}, SmpB\textsuperscript{LK}, and SmpB\textsuperscript{EK} showed a marked decrease in endogenous protein tagging activity (Fig. 2A, lanes 3–6). Compared with wild-type SmpB, the tagging propensity of these variants was reduced by 30, 50, and 65%, respectively (Fig. 2C). Maximum reduction in tagging activity was observed with a triple-alanine-substituted variant, E31A/L91A/K124A (SmpB\textsuperscript{ELK}), which showed a 70% loss of in vivo tagging activity (Fig. 2C). These findings demonstrate that several highly conserved residues, particularly residues Glu-31, Leu-91, and Lys-124, play an important role in trans-translation in vivo.

This Cluster of Conserved Residues Is Important for the in Vivo Association of the SmpB-SsrA Complex with 70 S Ribosomes—Having established that the highly conserved residues Glu-31, Leu-91, and Lys-124 play a key role in trans-translation, we wished to ascertain whether the decrease in endogenous tagging activity was due to a compromised ability of the SmpB variants to facilitate association of the SmpB-SsrA complex with stalled ribosomes. We have previously shown that the SmpB protein is required for stable association of SsrA RNA with 70 S ribosomes (14, 15). To assess the ribosome association propensities of the variants, we purified 70 S ribosomes from ΔsmpB cells harboring wild-type SmpB or one of its variants (SmpB\textsuperscript{EL}, SmpB\textsuperscript{EK}, SmpB\textsuperscript{LK}, and SmpB\textsuperscript{ELK}) and measured the levels of associated SmpB protein by Western blot analysis. This assessment revealed that all of the double- and triple-alanine-substituted SmpB variants had substantially decreased levels of SmpB protein associated with 70 S ribosomes (Fig. 3A, lanes 3–6). The most notable decrease was observed with double alanine variant SmpB\textsuperscript{EK} and the triple alanine variant SmpB\textsuperscript{ELK} (Fig. 3C).

One possible explanation for these findings could be that the substituted proteins are less stable and/or aggregate in the cell. This would lead to lower amounts of SmpB protein available to interact with SsrA RNA and mediate its association with 70 S ribosomes. To examine this possibility, we analyzed the levels of each protein in total cellular extracts and the S30 soluble fractions. We found that, like wild-type SmpB, the alanine-substituted proteins did not aggregate and were not present in the pellet fractions (data not shown). We found the SmpB\textsuperscript{E31A}, SmpB\textsuperscript{L91A}, SmpB\textsuperscript{K124A}, SmpB\textsuperscript{EL}, SmpB\textsuperscript{EK}, and SmpB\textsuperscript{LK} variants to be soluble, as wild-type levels of these substituted proteins were present in S30 extracts (Fig. 4, lanes 3–6, and data not shown). These results demonstrate that the ribosome association defects attributed to these variants were not due to a...
decrease in the total amount of SmpB protein present in the cells. These findings do suggest, however, that alanine substitutions of these highly conserved residues deter stable association of the SmpB protein with 70 S ribosomes.

Because SmpB function is required for stable association of SsrA RNA with stalled ribosomes, we probed for the amount of SsrA RNA in the same 70 S ribosome preparations. Consistent with our findings for SmpB protein, the level of 70 S ribosome-associated SsrA RNA was diminished in all SmpB variants tested (Fig. 5). The greatest decrease in the level of ribosome-associated SsrA RNA was observed with SmpBELK followed by SmpBEKL, SmpBLK, and SmpBEL (Fig. 5, A and C). This order corresponds precisely to the observed endogenous tagging phenotypes of these variants. Taken together, these data suggest that the ribosome association defects of the SmpB variants and the dramatic decrease in the levels of ribosome-associated SsrA RNA are most likely responsible for the deficiencies observed in the endogenous tagging assays (Fig. 2).

Alanine Substitution Variants of E. coli SmpB Protein Exhibit Defects in Binding SsrA RNA in Vitro—A critical first step in trans-translation is the binding of SmpB protein to SsrA RNA. Formation of a stable SmpB-SsrA complex is critical for the facilitated delivery of SsrA RNA to stalled ribosomes. Thus, the ability of SmpB to deliver SsrA to stalled ribosomes is directly related to the binding affinity and specificity of SmpB protein for SsrA RNA. To characterize this step in trans-translation, we generated expression constructs of SmpB protein carrying single, double, and triple alanine substitutions of key amino acid residues. Expression and purification of these substituted proteins followed the same basic procedure as for wild-type SmpB protein (see “Materials and Methods”). In all cases the substituted proteins were purified to greater than 95% homogeneity as detected by Coomassie-stained SDS-polyacrylamide gels. The apparent equilibrium dissociation constants ($K_d$) of each protein for interaction with SsrA113 (15) were determined using electrophoretic mobility-shift assay (see “Materials and Methods”). All binding assays were performed at least in triplicate, and $K_d$ values were derived by curve fitting using the equation SmpB$^{ELK}$, SmpB$^{LKL}$, and SmpB$^{EL}$ (Fig. 5, A and C).
$
\Theta_{eq} = C/(1 + K_d [SmpB])$, where $\Theta_{eq}$ is the fraction of RNA bound at equilibrium, $C$ is a constant representing the maximum fraction bound of the specific bound species, and $[SmpB]$ is the initial concentration of SmpB (15, 17). The binding assays were performed under highly stringent conditions to ensure the exclusive monitoring of high affinity and specificity binding interactions.

The single alanine substitution variants increase the equilibrium dissociation constants from 4 nM for wild-type SmpB protein to 20 nM for N93A, 29 nM for E31A, 38 nM for L91A, and 49 nM for K124A (see Table 1). Compared with wild-type SmpB, the N93A mutation reduces binding ~5-fold ($\Delta G = 1.0 \text{ kcal/mol}$), the E31A mutation reduces binding ~7-fold ($\Delta G = 1.2 \text{ kcal/mol}$), the L91A mutation reduces binding ~10-fold ($\Delta G = 1.4 \text{ kcal/mol}$), and the K124A mutation reduces binding ~12-fold ($\Delta G = 1.5 \text{ kcal/mol}$). Analysis of the single alanine substitution variants revealed that amino acids Asn-93, Glu-31, Leu-91, and Lys-124 contribute significantly to the binding of SmpB protein to SsrA RNA (Table 1). Amino acid residue Gln-94 does not contribute significantly to the binding between SmpB and SsrA, making comparatively marginal energetic contribution ($\Delta G = 0.3 \text{ kcal/mol}$) to complex formation. Clearly, each variant binds SsrA with significantly lower affinity than wild-type SmpB, as expected if these highly conserved residues in the wild-type protein make important contacts with SsrA RNA.

Next, we examined the SsrA binding affinities of several SmpB double alanine substitution variants, SmpB$^{E1L}$, SmpB$^{E1K}$, SmpB$^{L1K}$, and SmpB$^{NQ}$ (Fig. 6 and Table 1). Fig. 6 shows representative binding data and fitted curves for SmpB$^{WT}$, SmpB$^{E1L}$, and SmpB$^{E1K}$. When these single amino acid mutations were combined, we observed further additive losses in SsrA binding affinity, likely because of the loss of additional specific protein-RNA contacts. Compared with wild-type SmpB, the E31A/L91A double mutations (SmpB$^{E1L}$) reduce binding affinity ~10-fold ($\Delta G = 1.4 \text{ kcal/mol}$), the L91A/K124A mutations (SmpB$^{L1K}$) reduce binding affinity ~22-fold ($\Delta G = 1.8 \text{ kcal/mol}$), and the E31A/K124A mutations (SmpB$^{E1K}$) reduce binding affinity ~35-fold ($\Delta G = 2.1 \text{ kcal/mol}$). The greatest loss of binding affinity was observed with SmpB$^{L1K}$ and SmpB$^{E1K}$, which have $K_d$ values of 87 and 140 nM, respectively (Fig. 6, A and B, and Table 1). The triple alanine substitution variant E31A/L91/K124A (SmpB$^{L1K}$) further reduces binding affinity to ~44-fold ($\Delta G = 2.2 \text{ kcal/mol}$). Taken together, these data clearly demonstrate that the highly conserved amino acid residues Glu-31, Leu-91, and Lys-124 make substantial energetic contributions to binding of SmpB protein to SsrA RNA and participate in the formation of the ribonucleoprotein complex that recognizes stalled ribosomes.

T. tengcongensis SmpB binds SsrA RNA with high affinity—SmpB protein and SsrA RNA are conserved in eubacteria. Thus, it is reasonable to postulate that the specific contact residues implicated in the binding of E. coli SmpB protein to SsrA RNA might be similarly involved in the interactions of SmpB and SsrA orthologues from other bacterial species. To evaluate the merits of this hypothesis, we studied the interaction of Tten SmpB protein with Tten SsrA RNA. Toward
this end, we cloned, expressed, and purified wild-type Tten SmpB protein (Tten SmpBWT). The purification of Tten SmpB was greatly facilitated by its enhanced thermal stability. This protein is entirely soluble after a 10-min heat denaturation treatment at 65 °C that precipitates greater than 80% of soluble E. coli proteins (see “Material and Methods”). The Tten SmpB protein was purified to greater than 95% homogeneity as detected by Coomassie-stained SDS-polyacrylamide gels. We then performed gel mobility-shift assays to determine the binding affinity of Tten SmpBWT protein for Tten SsrA112. This Tten SsrA RNA variant, akin to the E. coli SsrA113 variant used for analysis of the E. coli SmpB-SsrA interactions, contains the tRNA-like domain along with a flanking segment linked via a short tetraloop. Electrophoretic mobility-shift assay analysis showed Tten SmpBWT to be capable of binding Tten SsrA RNA with high affinity, with a $K_d$ value of $\sim 12$ nM (Table 2). The binding assays were conducted in the presence of 300 mM KCl and 100-fold excess competitor RNA. The presence of a high salt concentration and more than a 100-fold excess of nonspecific competitor RNA indicates that the Tten SmpB binds Tten SsrA with highly affinity and specificity (Fig. 7).

**TABLE 2**

SsrA binding parameters, derived from curve-fitted gel mobility-shift data, for wild-type *T. tengcongensis* SmpB protein and alanine substitution variants

| *T. tengcongensis* SmpB | $K_d$ (nM) | $\Delta G$ (kcal mol$^{-1}$) | $\Delta \Delta G$ (kcal mol$^{-1}$) |
|------------------------|------------|------------------------------|----------------------------------|
| SmpBWT                 | 12 ± 2     | -10.8                        |                                  |
| SmpB$^{E28A}$          | 50 ± 6     | -9.9                         | 0.9                              |
| SmpB$^{D28A}$          | 120 ± 9    | -9.4                         | 1.4                              |
| SmpB$^{R90A}$          | 290 ± 18   | -8.9                         | 1.9                              |
| SmpB$^{L87A}$          | 350 ± 43   | -8.8                         | 2.0                              |
| SmpB$^{K120A}$         | 550 ± 50   | -8.5                         | 2.3                              |

**FIGURE 7.** SsrA binding assay with wild-type Tten SmpB protein. A, a representative autoradiogram of a gel mobility-shift assay, showing the binding propensity of Tten SmpBWT to Tten-SsrA112 RNA. B, curve-fit analysis of gel mobility-shift data with the Tten SmpBWT shown in panel A was used to determine its apparent equilibrium dissociation constant for binding to Tten SsrA (see Table 2).
stitution at Leu-87 decreased binding affinity for SsrA RNA by ~30-fold (ΔΔG = 2.0 kcal/mol). These data clearly demonstrate that the highly conserved surface residues of Tten SmpB protein, akin to the corresponding *E. coli* SmpB residues, play a crucial role in binding of SmpB protein to SsrA RNA.

**DISCUSSION**

This study was undertaken to investigate the contribution of conserved amino acid residues to the known functions of SmpB protein. The highly conserved nature and spatial distribution of amino acids Glu-31, Leu-91, and Lys-124 of SmpB protein suggested that these amino acids have been conserved for a functional reason (Fig. 1). In light of our biochemical studies it is now clear that these amino acids play a crucial role in SmpB binding to SsrA RNA.

The SmpB-SsrA quality control system is universally conserved in eubacteria. The relative simplicity of the SmpB-SsrA interaction and stability of the complex make it an ideal system for probing the basic biochemical tenets of RNA-protein interactions. It has been postulated that this system is essential for most, if not all, pathogenic bacteria since processes such as clearing stalled ribosomes and protein quality control may be even more critical under adverse conditions where errors leading to ribosome stalling are far more frequent. Indeed, recent reports suggest essential biological roles for the SmpB-SsrA system. For instance, analysis of mutants in the pathogenic bacteria *Neisseria gonorrhoeae*, *Mycoplasma pneumoniae*, and *Mycoplasma genitalium* suggest that SsrA activity is essential for viability (18, 19). Furthermore, the SmpB-SsrA system has been shown to play a crucial role in *Yersinia* pathogenesis. An *smpB-srrA* mutant of *Yersinia pseudotuberculosis* was shown to be avirulent and, thus, unable to cause mortality in mouse infection model. Additionally, the mutant had defects in survival within macrophages and suffered severe deficiencies in expression and secretion of *Yersinia* virulence effector proteins (20). Therefore, a better understanding of this unique and universal bacterial system might allow the design of highly specific new anti-bacterial agents.

The main objective of our studies was to gain a better understanding of the biochemical mechanism of how SmpB protein recognizes SsrA RNA and promotes the detection and rescue of stalled ribosomes. When comparing the amino acid conservation profiles of SmpB protein, we found intriguing patterns of conservation for certain residues. Of the 115 sequences analyzed, the *E. coli* SmpB residues Glu-31, Leu-91, and Lys-124 enjoy a high degree of conservation, 93, 100, and 99%, respectively. Interestingly, single alanine substitution of each residue diminished the propensity of the SmpB protein to support the endogenous tagging activity (Fig. 2). Several combinations of double alanine substitutions of these residues yielded further insights into the significance of these residues. Contributions made by these residues to SmpB function became more evident by the substantial decline in their ability to tag endogenous substrates, with the double-alanine-substituted variants SmpBEL, SmpBLK, and SmpBEK, which exhibited tagging deficiencies of 30, 50, and 65%, respectively. Amino acid residues Asn-93 and Gln-94 are only marginally conserved among SmpB orthologues, 16 and 7%, respectively. Correspondingly, single or double alanine substitutions at these two residues make negligible contribution to the endogenous tagging activity of the *E. coli* SmpB protein.

Analysis of 70 S ribosomes from the mutant strains provided additional insights into the nature of the defect exhibited by the SmpB variants. The substantial reduction in the levels of 70 S-associated SmpB protein and SsrA RNA led us to conclude that the trans-translation defects linked most prominently with the SmpBEL, SmpBLK, and SmpBEK variants were due to their inability to effectively deliver SsrA RNA to stalled ribosomes (Fig. 5). One possible explanation for the ribosome association defect could be that these resi-
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dues make base specific contact with SsrA RNA. Loss of these specific contacts in the variants might then reduce SmpB binding affinity for SsrA RNA and adversely affect the delivery of SsrA RNA to stalled ribosomes. Indeed, biochemical studies using gel mobility-shift analysis confirm this conclusion, demonstrating that the alanine-substituted proteins exhibit substantial SsrA binding defects. It is interesting to note that SmpB variants with the greatest defect in SsrA binding also exhibit the most severe ribosome association defects, suggesting that the in vivo association of SmpB protein with stalled 70 S ribosomes is dependent on its productive and stable interaction with SsrA RNA.

Mutations of the equivalent *T. tengcongensis* amino acids (Glu-28, Leu-87, His-89, Arg-90, and Lys-120) also severely reduced the binding affinity of Tten SmpB protein for Tten SsrA RNA, suggesting that a similar RNA binding surface is present in all SmpB protein analogues. Contribution to SsrA binding affinity made by amino acid residues Glu-28, Leu-87, His-89, Arg-90, and Lys-120 of *T. tengcongensis* also correlate well with the degree of conservation of these specific residues. *T. tengcongensis* SmpB residues Glu-28, Leu-87, and Lys-120, which make the most significant contributions to SsrA binding affinity, are also each greater than 93% conserved among eubacterial SmpB orthologues. Most interestingly, Tten SmpB amino acids His-89 and Arg-90, which are, respectively, 66 and 33% conserved, make greater energetic contributions to SsrA binding affinity of Tten SmpB than the corresponding less conserved *E. coli* SmpB residues Asn-93 and Gln-94, suggesting perhaps specialized co-evolution of specific SmpB-SsrA pairs in each bacterial species.

Our studies were greatly aided by a number of previously described in vivo and in vitro activity assays (15). These assays have been instrumental in deciphering the functional significance of a number of highly conserved and semiconserved amino acid residues in SmpB protein function. The in vivo activity assays, endogenous protein tagging and ribosome association, depend on continuous low level expression of the SmpB protein or its variants from a multicopy number plasmid (see “Materials and Methods” for details). We suspect that the readout of these in vivo assays most likely represents an underestimation of the functional contribution of individual amino acids to the activity of SmpB protein. Because all SmpB alanine substitution variants retain some degree of RNA binding activity, a greater than physiological intracellular concentration of the protein from a multicopy number plasmid would shift the equilibrium toward the RNA-bound form and, hence, give an under-representation of the in vivo contribution of each residue to the overall SmpB function.

At its inception, our investigation was guided solely by the degree of sequence conservation of specific amino acids and their contribution to biological function, as no structural data for the SmpB protein were yet available. Recently, a great deal of structural information regarding SmpB and SsrA has become available. Solution NMR structures of SmpB protein from *Aquifex aeolicus* and *Thermus thermophilus* along with a co-crystal structure of *A. aeolicus* SmpB in complex with the tRNA-like domain of SsrA have been solved (21–23). The core SmpB structure is quite similar in all three structural models. The biochemical and structural data on SmpB protein (14, 21–23) and SmpB-SsrA complex (22) have confirmed the utility and validity of our approach. Indeed NMR structural models place residues Glu-31, Leu-91, Asn-93, and Lys-124 in close spatial proximity to each other and clustered on an exposed surface of the protein (21). The SmpB-SsrA co-crystal structure places these residues provocatively close to the potential D-arm of SsrA RNA. However, at the current level of resolution, 3.2 Å, the individual contribution of each amino acid to the SsrA binding activity of SmpB protein cannot be readily ascertained. The biochemical studies described herein are entirely consistent with these structural studies. Moreover, our biochemical investigations provide direct calculation of the relative importance and energetic contributions of each highly conserved amino acid to interactions of SmpB protein with SsrA RNA. In summary, our findings expand the conceptual framework provided by the recent structural studies and provide strong biochemical evidence for the crucial role of highly conserved residues Glu-31, Leu-91, Asn-93, and Lys-124 in binding of SmpB protein to SsrA RNA.

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