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SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA)

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In bacteria, SsrA RNA recognizes ribosomes stalled on defective messages and acts as a tRNA and mRNA to mediate the addition of a short peptide tag to the C-terminus of the partially synthesized nascent polypeptide chain. The SsrA-tagged protein is then degraded by C-terminal-specific proteases. SmpB, a unique RNA-binding protein that is conserved throughout the bacterial kingdom, is shown here to be an essential component of the SsrA quality-control system. Deletion of the *smpB* gene in *Escherichia coli* results in the same phenotypes observed in *ssrA*-defective cells, including a variety of phage development defects and the failure to tag proteins translated from defective mRNAs. Purified SmpB binds specifically and with high affinity to SsrA RNA and is required for stable association of SsrA with ribosomes *in vivo*. Formation of an SmpB–SsrA complex appears to be critical in mediating SsrA activity after aminoacylation with alanine but prior to the transpeptidation reaction that couples this alanine to the nascent chain. SsrA RNA is present at wild-type levels in the *smpB* mutant arguing against a model of SsrA action that involves direct competition for transcription factors.

Keywords: 10Sa RNA/phage development/protein degradation/ribosome association/translation

Introduction

SsrA RNA, also known as 10Sa RNA or tmRNA, is encoded in 12 of the 13 completely sequenced bacterial genomes as well as in many additional bacteria whose genome sequences are incomplete (Ushida *et al*., 1994; Williams and Bartel, 1996, 1998). The inclusion of this RNA of ~360 nucleotides in even the limited genetic repertoire of *Mycoplasma genitalium* (482 genes) as well as in Gram-negative and Gram-positive species suggests an important biological role. Nevertheless, mutation of the *ssrA* gene in *Escherichia coli* results in a viable strain. SsrA-defective strains have a variety of subtle phenotypes including temperature-sensitive growth, reduced motility, inability to support growth of λ*imm*P22 hybrid phage, induction of Alp protease activity and enhanced activity of several repressor proteins (Kirby *et al*., 1994; Komine *et al*., 1994; Retallack *et al*., 1994; Retallack and Friedman, 1995).

The SsrA molecule has features of both a tRNA and an mRNA, including the ability to be charged with alanine, bind 70S ribosomes and to encode sequences found at the C-termini of some intracellular proteins (Komine *et al*., 1994; Ushida *et al*., 1994; Tu *et al*., 1995; Felden *et al*., 1996, 1997; Keiler *et al*., 1996; Muto *et al*., 1996, 1998; Tadaki *et al*., 1996; Williams and Bartel, 1996, 1998). The dual role of SsrA as a tmRNA was first suggested in a model in which alanine-charged SsrA RNA binds in the A site of ribosomes stalled on damaged mRNA, donates the charged alanine to the nascent polypeptide chain and then replaces the damaged message as a surrogate mRNA, directing translation of a short peptide tag encoded by a sequence internal to SsrA (Keiler *et al*., 1996). The final translation product of this process carries an 11-residue C-terminal peptide tag (AANDENYALAA) that makes the tagged protein a substrate for proteolysis by C-terminal specific proteases (Tu *et al*., 1995; Keiler *et al*., 1996; Gottesman *et al*., 1998). This system prevents the accumulation of ribosomes stalled at the end of damaged mRNAs that lack a stop codon and also provides a general quality-control system that degrades incomplete protein fragments which otherwise might have inappropriate cellular activities.

The current understanding of SsrA function is based largely on the tRNA-like properties of SsrA RNA and on the structures of tagged protein products. It is not known whether additional cellular factors are required for SsrA function or how the diverse phenotypes of SsrA-defective cells are related to the activities of SsrA. In *Salmonella typhimurium*, insertion mutations in *smpB* (small protein B), a gene immediately upstream of *ssrA*, prevent plating of bacteriophage P22 but not *c*1^{-} mutants of P22 (M.M.Susskind and K.Hughes, unpublished data). This phenotype is the same as that originally reported by Retallack *et al*. (1994) for plating of λ*imm*P22 hybrid phage on SsrA-defective *E.coli*. Disruption of the *smpB* gene was previously reported to decrease the virulence of *S.typhimurium* in mice and to reduce bacterial survival within macrophages (Bäumler *et al*., 1994). Here, we demonstrate that deletion of the *smpB* gene in *E.coli* prevents SsrA-mediated peptide tagging and mimics other phenotypes of SsrA-deficient strains. We also show that SmpB is an RNA-binding protein that binds specifically to SsrA and that SmpB is required for stable association of SsrA with ribosomes in the cell.

SsrA RNA is required for phage λ*imm*P22 development and has been proposed to prevent phage growth by competing with DNA sites for binding of transcription factors (Retallack and Friedman, 1995). We find, however,

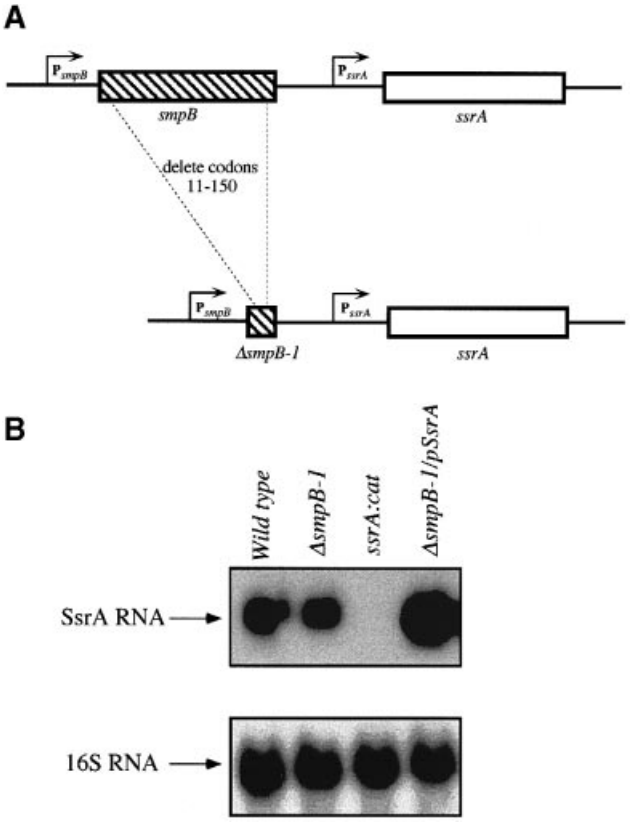

Fig. 1. (**A**) The *smpB* and *ssrA* genes are arranged tandemly in *E.coli* and *S.typhimurium*, although these genes are not adjacent in many other bacterial genomes. Δ*smpB-1* is an in-frame deletion of the *E.coli* SmpB coding sequence. (**B**) Northern blot probed with ^{32}P-labeled DNA oligonucleotides complementary to SsrA RNA (top) or 16S ribosomal RNA (bottom) in strains W3110, W3110 Δ*smpB-1*, W3110 *ssrA::cat* and W3110 Δ*smpB-1*/pSsrA.

that SsrA RNA is processed and present at normal levels in the *smpB* mutant and yet this strain still fails to plate λ*imm*P22. This result indicates that SsrA cannot affect phage development by a simple competition mechanism.

Results

SmpB mutants have the same phenotypes as SsrA mutants

To address the possible functions of the SmpB protein in SsrA-mediated protein tagging, we constructed an SmpB-defective strain by deleting >80% of the *smpB* gene in the W3110 strain of *E.coli* (Figure 1A). This in-frame deletion (Δ*smpB-1*) does not alter the normal initiation and termination signals for *smpB* transcription and translation. The parental strain (W3110) and the otherwise isogenic Δ*smpB-1* and *ssrA::kan* strains displayed no differences in growth rate or colony morphology at temperatures ranging from 16 to 42°C, on rich or minimal media. At temperatures >42°C, the Δ*smpB-1* and *ssrA::kan* strains had slightly increased doubling times (~10%) in comparison with the parental strain. Temperature-sensitive growth of W3110 *ssrA::kan* has been reported previously (Komine *et al*., 1994).

We first tested whether SmpB is required for the synthesis of SsrA RNA. Northern blot hybridization experiments (Figure 1B) show that SsrA RNA is present at wild-type levels in Δ*smpB-1* cells and is overproduced in Δ*smpB-1* cells transformed with a plasmid bearing the *ssrA* gene. Moreover, in this and other experiments using gels capable of ±1 nucleotide resolution, SsrA RNA from Δ*smpB-1* cells had the same electrophoretic mobility as SsrA from wild-type cells, indicating that SmpB is not required for the 3′ and 5′ processing events that convert precursor SsrA RNA into its mature, active form (Subbarao and Apirion, 1989; Komine *et al*., 1994; Li *et al*., 1998).

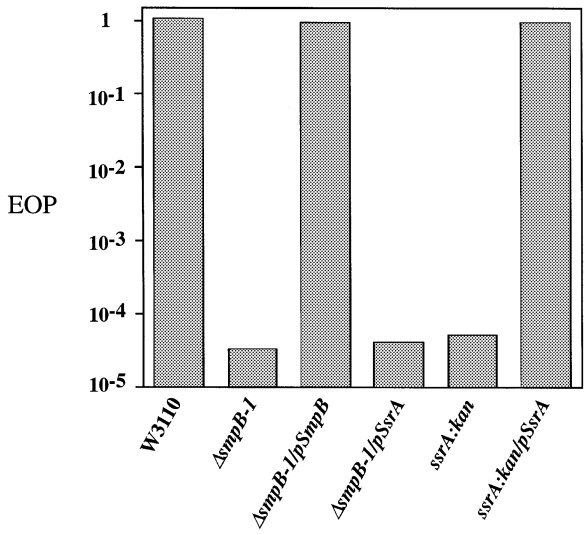

Fig. 2. Plating efficiency of phage λ*imm*P22 *dis c*2-5 on wild-type (W3110), SmpB-defective (W3310 Δ*smpB-1*) and SsrA-defective (W3310 *ssrA::kan*) strains with or without plasmids expressing SmpB (pSmpB) or SsrA (pSsrA).

Some of the most distinctive phenotypes of SsrA-defective strains involve phage development. For example, certain λ*imm*P22 hybrid phage fail to plate efficiently on *E.coli* strains lacking functional SsrA (Retallack *et al*., 1994) and, as we now show, SmpB-defective strains are similarly compromised. The plating efficiency of λ*imm*P22 *dis c*2-5 on Δ*smpB-1* cells was reduced by four orders of magnitude to a level comparable with that observed with *ssrA::kan* cells (Figure 2). This defect in λ*imm*P22 plating on Δ*smpB-1* cells could be complemented by a low copy-number plasmid expressing the *smpB* gene but not by a plasmid that overexpresses SsrA RNA (Figure 2). Thus, λ*imm*P22 fails to plate efficiently on the Δ*smpB-1* strain because these cells lack the SmpB protein.

Phage Mu carrying a temperature-sensitive variant of Mu *c* repressor (*c-ts*) cannot be induced to grow lytically in lysogenic strains lacking SsrA RNA (T.Baker, personal communication). Figure 3 shows that a Mu *c-ts* lysogen of the wild-type strain lysed ~1 h after a temperature shift to 42°C, whereas *c-ts* lysogens of the Δ*smpB-1* and *ssrA::kan* strains failed to lyse and bacterial growth continued at normal rates. This defect in phage Mu induction in Δ*smpB-1* and *ssrA::kan* strains was complemented by low copy-number plasmids expressing SmpB and SsrA, respectively. Thus, the Δ*smpB-1* and *ssrA::kan* strains both fail to support induction of the lytic development of Mu *c-ts* lysogens.

SmpB is required for SsrA-mediated peptide tagging

SsrA RNA is essential for the C-terminal addition of degradation tags to polypeptides synthesized from mRNAs

lacking a stop codon (Keiler *et al*., 1996). To investigate whether SmpB protein is required for this process, we used a gene (λ-*M2-H6-trpAt*) that encodes the N-terminal domain of λ repressor with M2 epitope and His6 sequences and a trpAt transcriptional terminator (Keiler *et al*., 1996). The mRNA transcribed from this gene has no in-frame stop codons, and, in wild-type cells, the λ-M2-H6-trpAt protein is tagged and degraded. Western blots showed that the λ-M2-H6-trpAt protein was present at equivalent levels in both Δ*smpB-1* and *ssrA::kan* cells (Figure 4A, lanes 1 and 2) but did not accumulate in wild-type cells or in a Δ*smpB-1* strain in which SmpB was provided from a plasmid (Figure 4, lanes 3 and 4). These differences in steady-state protein levels are expected if the λ-M2-H6-trpAt protein is tagged and degraded in cells containing both SmpB and SsrA but is stable in cells lacking either SmpB or SsrA because the *ssrA*-encoded degradation tag is not added.

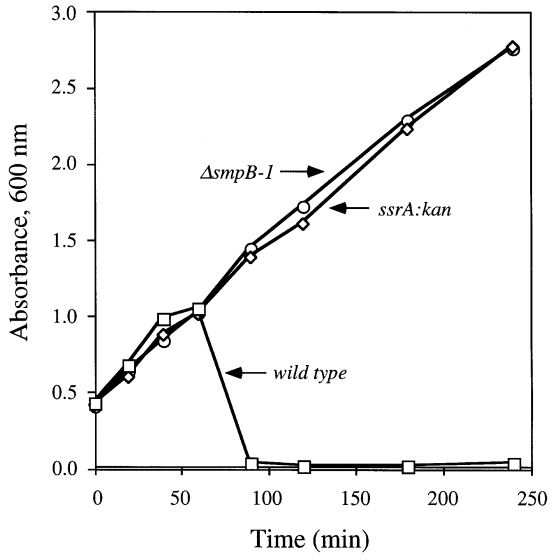

Fig. 3. Time course of induction of phage Mu *c-ts62 pAp1* in lysogens of wild-type (W3110), SmpB-defective (W3310 Δ*smpB-1*) and SsrA-defective (W3310 *ssrA::kan*) strains following a 30–42°C temperature shift to inactivate the Mu *c-ts62* repressor. The decrease in absorbance at 600 nm is caused by phage-induced cell lysis.

To monitor directly for SsrA-mediated tagging, we characterized the tagging of the λ-M2-H6-trpAt protein in a strain containing the SsrA^{DD} RNA variant, which mediates the addition of a peptide tag (AANDENYALDD) that does not result in degradation (E.Roche and R.Sauer, unpublished). Proteins modified with this mutant tag can be detected using antibodies to the DD-peptide tag. Each of the strains in lanes 5–7 of Figure 4 contains the λ-*M2-H6-trpAt* gene and SsrA^{DD} RNA but only the strain lacking SmpB failed to accumulate the DD-tagged λ-M2-H6-trpAt protein (Figure 4, lane 5, lower panel). Untagged λ-M2-H6-trpAt protein was, however, expressed in this *smpB*$^{-}$ strain (Figure 4, lane 5, upper panel) indicating that the defect is in SsrA^{DD}-mediated tagging of λ-M2-H6-trpAt rather than in its synthesis. Overall, these results provide strong evidence that the SmpB protein is required for efficient SsrA-mediated tagging of proteins synthesized from mRNAs that lack in-frame termination codons.

The λ-M2-H6-trpAt proteins were purified using Ni-NTA chromatography and analyzed by mass spectrometry (data not shown). The proteins from strains lacking functional SmpB or SsrA had masses within 1 Da of that predicted for untagged λ-M2-H6-trpAt protein lacking the N-terminal formyl methionine (residues 2–117). The proteins purified from strains containing both SmpB and SsrA^{DD} had masses expected for residues 2–117 of λ-M2-H6-trpAt plus the AANDENYALDD tag. In strains lacking SmpB, no addition of alanine or any part of the SsrA-degradation tag to the λ-M2-H6-trpAt protein was detected, although the possibility that a small amount of protein is tagged and then degraded cannot be absolutely excluded. We note, however, that similar quantities of untagged λ-M2-H6-trpAt protein were expressed in the Δ*smpB-1* and *ssrA::kan* strains (Figure 4, lanes 1 and 2).

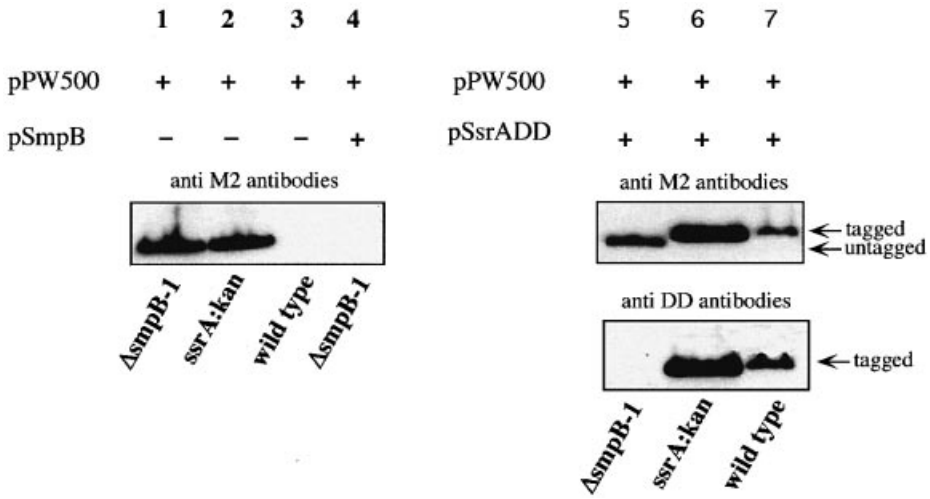

Fig. 4. λ-M2-H6-trpAt protein, expressed from an mRNA with no stop codon, accumulated in cells lacking SmpB or SsrA (lanes 1 and 2) but not in cells containing functional SmpB and SsrA (lanes 3 and 4). λ-M2-H6-trpAt protein was detected by immunoblotting with anti-FLAG M2 antibodies or anti-DD-tag antibodies. Comparison of the upper and lower panels in lanes 5–7 indicates that the accumulation of tagged λ-M2-H6-trpAt protein in cells expressing SsrA^{DD} required SmpB. Plasmid pPW500 encodes λ-M2-H6-trpAt; SsrA^{DD} encodes the protease-resistant AANDENYALDD tag.

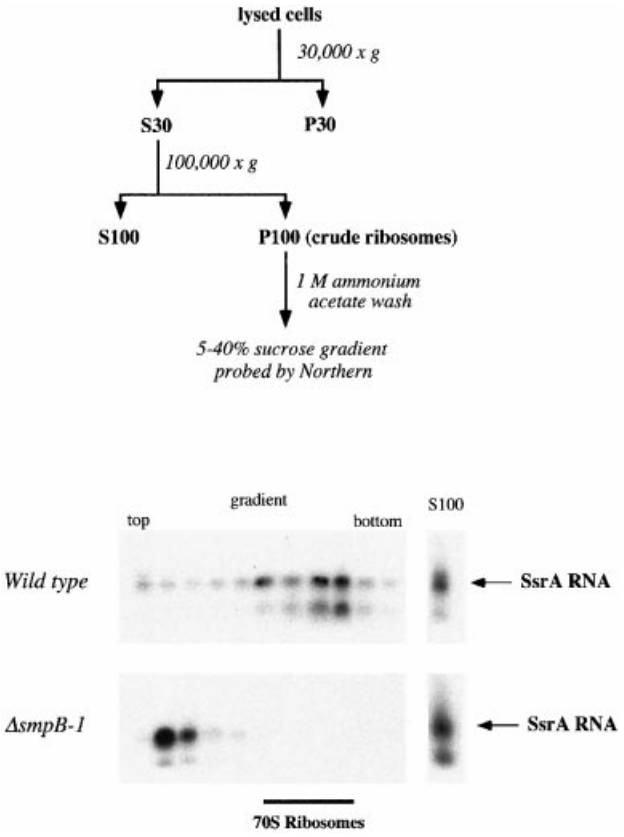

Fig. 5. SsrA RNA co-sediments with 70S ribosomes in lysates of wild-type but not Δ*smpB-1* cells. (**A**) W3110 and W3110 Δ*smpB-1* cell lysates were fractionated using the centrifugation and washing steps shown (Komine *et al*., 1996). (**B**) The presence of SsrA RNA in each fraction was assayed by Northern blot hybridization using ^{32}P-labeled oligonucleotides complementary to SsrA RNA. The lower band appears to be an SsrA degradation product. The position of 70S ribosomes in the gradient was determined by probing with oligonucleotides complementary to 16S RNA.

Hence, if tagging does occur in the Δ*smpB-1* strain, then it must be inefficient.

SmpB protein is required for SsrA RNA association with ribosomes

In lysates prepared from wild-type cells, SsrA RNA co-fractionates with 70S ribosomes (Komine *et al*., 1996; Tadaki *et al*., 1996). To test whether SmpB is required for stable association of SsrA with ribosomes, we fractionated cell lysates prepared from wild-type and Δ*smpB-1* cells and monitored the localization of SsrA RNA by Northern blot hybridization. As expected from previous work (Komine *et al*., 1996; Tadaki *et al*., 1996), SsrA RNA in wild-type cell lysates co-sedimented with 70S ribosomes near the bottom of the final sucrose gradient (Figure 5). By contrast, in Δ*smpB-1* cells SsrA was found in the top few fractions of the sucrose gradient and was not associated with ribosomes. Furthermore, a greater fraction of the total SsrA RNA was recovered in the S100 fraction in Δ*smpB-1* cells than in wild-type cells. Taken together, these results suggest that SmpB protein directly or indirectly facilitates the stable association of SsrA RNA with 70S ribosomes.

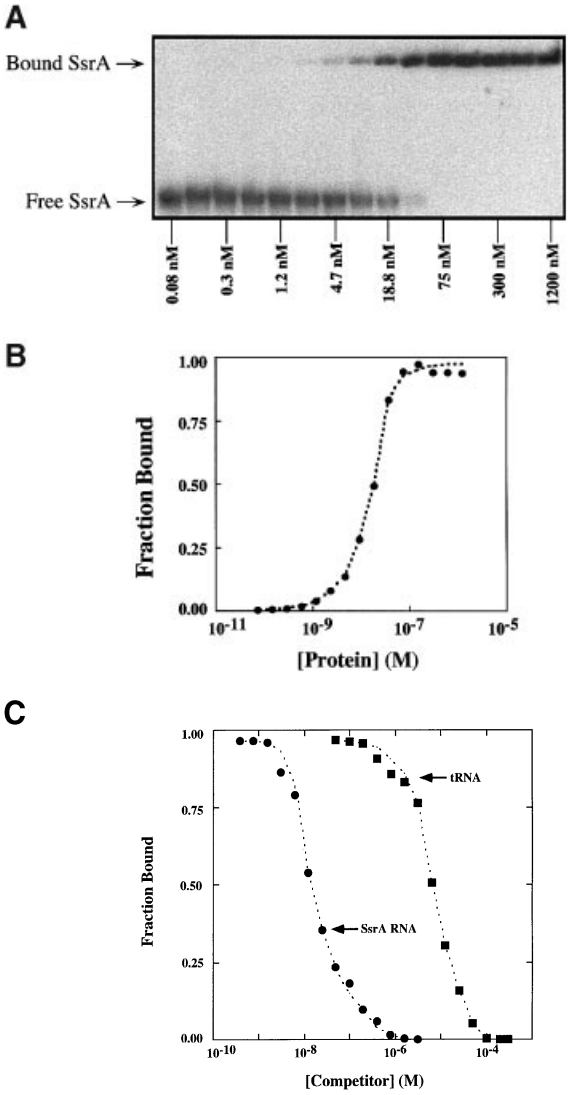

Fig. 6. (**A**) Gel-mobility shift assay of the binding of purified SmpB to 100 pM of ^{32}P-labeled SsrA RNA produced by transcription *in vitro*. (**B**) Quantified binding data from (A). (**C**) Competition for binding of SmpB to 100 pM ^{32}P-labeled SsrA by unlabeled SsrA RNA or total yeast tRNA.

SmpB protein binds SsrA RNA

To test directly whether SmpB protein interacts with SsrA RNA, the protein was overexpressed, purified and characterized *in vitro*. Sequential Edman degradation of the purified protein gave the N-terminal sequence TKKKAHK as expected for *bona fide* SmpB after removal of the initiator formyl methionine. Purified SmpB behaved as a stably folded protein, displaying cooperative denaturation and a far-UV circular dichroism (CD) spectrum indicative of a predominantly β-sheet protein (data not shown).

The interaction of SmpB protein with SsrA RNA was investigated using a gel-mobility shift assay. As shown in Figure 6A, addition of increasing concentrations of SmpB resulted in formation of an SmpB–SsrA RNA complex. The observed binding is saturable with half-maximal binding at a free SmpB concentration of ~20 nM. To probe the specificity of SmpB–SsrA binding, unlabeled SsrA RNA and total yeast tRNA were used to compete for binding of 200 nM SmpB to 100 pM ^{32}P-labeled

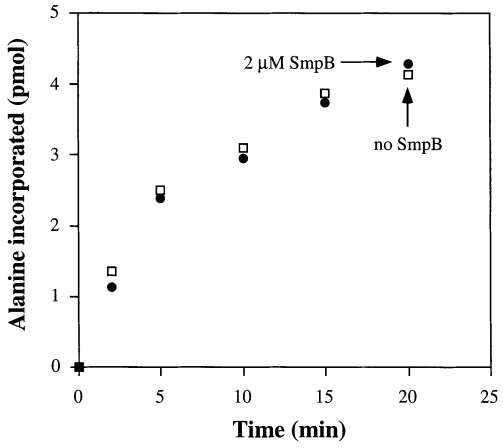

Fig. 7. Kinetics of charging of SsrA RNA (18 pmol) produced by transcription *in vitro* with [^{3}H]alanine by purified alanyl-tRNA synthetase in the presence and absence of SmpB.

SsrA (Figure 6C). Approximately 400-fold higher molar concentrations of yeast tRNA than SsrA RNA were required to achieve the same degree of competition. These results demonstrate that SmpB is an RNA-binding protein with a high degree of affinity and specificity for SsrA RNA.

SmpB does not affect charging of SsrA

SsrA can be charged with alanine by alanyl-tRNA synthetase *in vitro* (Komine *et al*., 1994; Ushida *et al*., 1994). As shown in Figure 7, SsrA RNA produced by transcription *in vitro* was charged with alanine by purified alanyl-tRNA synthetase at the same rate in the presence and absence of 2 μM SmpB, a concentration that results in complete binding in the gel-mobility shift assay. Thus, SmpB protein is neither required for nor significantly affects SsrA charging. Furthermore, SsrA RNA purified from wild-type and Δ*smpB-1* cells could be charged with ^{3}H-alanine by alanyl-tRNA synthetase with similar kinetics (data not shown), providing additional evidence that SsrA RNA is properly processed in SmpB-defective cells.

Discussion

The *E.coli* SmpB protein contains 160 amino acids and is reasonably basic (36 Lys, His and Arg residues; 18 Asp and Glu residues). Homology searches using PSI-BLAST (Altschul *et al*., 1997) revealed related sequences with pairwise identities from 32 to 78% in all 13 bacterial species with known genome sequences. Interestingly, *Rickettsia prowazeki* contains an SmpB homolog but no identified SsrA homolog (Williams, 1999), suggesting either that SmpB may also have SsrA-independent roles or that SsrA orthologs are very difficult to identify in some species. Sequence searches failed to detect significant homologies between the SmpB family and any other bacterial proteins or with eukaryotic or archaeal proteins. Thus, SmpB appears to play a sufficiently important biological role to be universally present in bacteria and may constitute a unique protein family.

Escherichia coli strains lacking the SmpB protein have the same phenotypes as strains lacking SsrA RNA, suggesting that SmpB is essential for SsrA function. Both SmpB-defective and SsrA-defective strains fail to add any part of the AANDENYALAA degradation tag to proteins synthesized from damaged mRNA, and do not support growth of λ*imm*P22 hybrid phage or induction of Mu *c-ts* phage. SmpB-defective strains contain normal levels of SsrA RNA and are not complemented by SsrA-overproducing plasmids, indicating that the absence of SsrA-mediated function in these strains is not caused by a deficiency of SsrA RNA. Finally, SmpB is not required for the processing of SsrA RNA to its mature and active form.

SmpB protein binds to SsrA RNA *in vitro* with an affinity (~20 nM) that would ensure substantial binding at the concentrations of SsrA that have been measured *in vivo* (~10 μM; Altuvia *et al*., 1997). Because our binding studies were performed with SsrA RNA transcribed *in vitro*, the base modifications that occur *in vivo* (Felden *et al*., 1998) cannot be essential for formation of the SmpB–SsrA complex. Retallack and Friedman (1995) have reported that SsrA RNA binds to a number of bacterial repressor and activator proteins in low-salt buffer (50 mM KCl), raising the question of whether the SmpB–SsrA interaction that we observe is specific. In competition experiments, however, SmpB binds to SsrA RNA ~400-fold more tightly than to bulk tRNA, indicating that this interaction is specific with respect to the identity of the bound RNA. In addition, the binding assays shown in Figure 6 were performed in buffer containing 200 mM KCl, an ionic strength comparable with or higher than physiological. Finally, the fact that SmpB-defective cells are also defective in SsrA function makes it exceedingly likely that the SmpB–SsrA interaction observed *in vitro* is biologically significant.

The ability of SsrA to function as both a tRNA and an mRNA has been proposed to require charging with alanine, recognition of the A site of ribosomes in which mRNA translation has stalled, participation in the transpeptidation reaction that adds the charged alanine to the growing nascent chain, movement to the P site and finally the ability to release the stalled mRNA and to serve as a message that directs translation of the last 10 residues of the degradation tag (Keiler *et al*., 1996). Regardless of the model, the results presented here indicate that SmpB is not required for charging of SsrA but is required to permit SsrA RNA to associate stably with 70S ribosomes. Moreover, because no tagging or addition of alanine to the λ-M2-His6-trpAt protein was detected in cells lacking SmpB, it is possible that SmpB is required for some tRNA-like function of SsrA following charging but prior to the transpeptidation reaction.

A ternary complex of elongation factor Tu (EFTu), GTP and aminoacylated tRNA protects the labile ester linkage between normal tRNAs and their charged amino acids and mediates binding to the A site of the ribosome (for review, see Sprinzl, 1994). Although SmpB is not homologous to EFTu, it might serve one or both of these EFTu-like functions for SsrA. However, EFTu–GTP binding to tRNA mainly involves sugar–phosphate backbone contacts in the acceptor and TΨC stems (Nissen *et al*., 1995) and because SsrA appears to contain these tRNA-like elements of secondary structure (Komine *et al*., 1994; Felden *et al*., 1996; Williams and Bartel, 1996), it is plausible that EFTu itself could serve these functions for SsrA. Another possible role for SmpB would be to

serve as a protein mimic of the anticodon helix and loop of a normal tRNA, in a manner analogous to domain 4 of elongation factor G (Ævarsson *et al*., 1994; Czworkowski *et al*., 1994; Nissen *et al*., 1995). SsrA is not believed to have an anticodon helix or to require codon–anticodon interactions for ribosome binding. If SmpB acts as an anticodon–helix mimic, it might provide additional contacts required for high-affinity ribosome binding, regulate the GTPase activity of a ribosome-bound EFTu–GTP–SsrA complex or mediate interactions with ribosomal elements required for translocation.

The regulatory activities of proteins such as P22 C1, Lac repressor, LexA repressor and λ repressor are increased in SsrA-defective cells (Retallack *et al*., 1993, 1994; Retallack and Friedman, 1995). This observation explains many of the phenotypes of SsrA-defective cells. For example, hyperactivity of the Mu *c-ts* repressor in SsrA-defective cells could explain why phage Mu induction is not observed in SsrA-defective cells. Enhanced activity of the P22 C1 activator could lead to excess transcription from the p_{aQ} promoter in λ*imm*P22 (Hoopes and McClure, 1985; Ho and Rosenberg, 1985; Stephenson, 1985; Ho *et al*., 1992), resulting in interference with lytic phage development and explaining the inability of this phage to plate on SsrA-defective strains. λ*imm*P22 phage that are $c1^-$ or that have an altered-specificity C1, which would not be expected to activate p_{aQ}, do plate on SsrA-defective strains (Strauch *et al*., 1986; Retallack *et al*., 1993, 1994). Purified SsrA RNA binds P22 C1, Lac repressor, LexA repressor and λ repressor *in vitro*, and it has been proposed that SsrA RNA also binds these regulatory proteins *in vivo*, reducing their free concentrations and thereby diminishing their biological activities (Retallack and Friedman, 1995). Our results, however, cast doubt on this binding model because wild-type SsrA RNA is present at normal levels in Δ*smpB-1* strains and at higher than normal levels in Δ*smpB-1* pSsrA strains, and yet both of these strains have the same phage development phenotypes as SsrA-defective strains. It is not known how the SmpB–SsrA system does function to increase the activities of proteins like P22 C1 and Mu *c-ts* repressor.

A number of questions remain to be answered. Are macromolecules in addition to SmpB required for SsrA function? How does SmpB binding support SsrA-mediated peptide tagging? Does SmpB have functions independent of SsrA? The identification of SmpB as an essential participant in SsrA function represents an important step forward that should help to guide future studies of detailed mechanism and biological function in the SmpB–SsrA peptide-tagging system.

Materials and methods

Strains, plasmids and phage

Escherichia coli strains were grown in Luria–Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 5 g NaCl/l) or on LB–agar plates containing 1–1.5% agar/l. Ampicillin (100 μg/ml) and/or tetracycline (30 μg/ml) were added to plates or media as needed. Plasmid pKW1 and its derivatives, pKW11 (pSsrA) and pKW23 ($pSsrA^{DD}$), are tetracycline-resistant plasmids with pACYC replicons (K.Williams and E.Roche, unpublished). Plasmid pPW500 (ampicillin resistance; colE1 replicon) directs transcription of an mRNA with no in-frame stop codons that encodes the N-terminal domain of λ repressor with an M2 FLAG epitope and His6 tag (Keiler *et al*., 1996). To generate plasmid pSmpB, the promoter and coding sequences of the *smpB* gene were amplified by PCR to add *Nco*I and *Eco*RI sites at the 5′ and 3′ ends, respectively. This DNA was digested with *Nco*I and *Eco*RI and cloned into *Nco*I–*Eco*RI-digested pKW1. The method described by Link *et al*. (1997) was used to generate a precise deletion of the *smpB* gene, first in a derivative of plasmid pKO3 and then in the chromosome of *E.coli* strain W3110. The structure of the chromosomal deletion (Δ*smpB-1*) was verified by PCR sequencing. Strain W3110 *ssrA::kan* was a gift of Hachiro Inokuchi; in this strain, a portion of the promoter and approximately two-thirds of the *ssrA* gene have been replaced with a kanamycin-resistance gene (Komine *et al*., 1994). Strain W3110 *ssrA::cat* was constructed by phage P1 transduction from strain X90 *ssrA::cat* (Keiler *et al*., 1996). The plating efficiency of phage λ*imm*P22 *dis c*2-5 (a gift from David Friedman) on the wild-type strain W3110 and its otherwise isogenic derivatives W3110 Δ*smpB-1* and W3110 *ssrA::kan* was determined as described previously (Retallack *et al*., 1994). Phage Mu *c-ts62 pAp1* was a gift of Tania Baker; Mu *c-ts62 pAp1* lysogens of strain W3110 were selected by ampicillin resistance.

Overexpression and purification of SmpB protein

The coding sequence of the *smpB* gene of *E.coli* was amplified by PCR to add a *Nde*I site at the 5′ end and a *Bam*HI site at the 3′ end. This DNA fragment was cloned into the *Nde*I–*Bam*HI digested pET9a expression vector (Novagen) to generate the SmpB-overexpressing plasmid pSmpB9a. High levels of SmpB protein were expressed from pSmpB9a but were largely present in inclusion bodies. As a result, we purified SmpB under denaturing conditions and refolded the purified protein.

Escherichia coli strain BL21(DE3)/pLysS/pSmpB9a was grown in 6 l of LB broth at 37°C to an A_{600} of 0.6, induced with 1 mM IPTG and grown for an additional 3 h. The cells were harvested by centrifugation, resuspended in 150 ml of buffer A [50 mM HEPES pH 7.5, 5 mM dithiothreitol (DTT), 1 mM EDTA and 8 M urea] and lysed by sonication. The cell lysate was centrifuged at 15 000 r.p.m. in a Sorvall SS-34 rotor to remove cellular debris, and the supernatant was loaded on a Source™ 15S column (Pharmacia Biotech). The column was washed with buffer A and eluted with a linear gradient from 0 to 600 mM KCl in buffer A. SmpB protein eluted from the column between 210 and 250 mM KCl. Fractions containing SmpB protein were combined and loaded directly onto a C4 reverse-phase HPLC column (Vydak™) equilibrated in 30% acetonitrile and 0.1% trifluoroacetic acid. A linear gradient from 36 to 46% acetonitrile in 0.1% trifluoroacetic acid was applied and SmpB-containing fractions were combined and lyophilized. The protein was resuspended in buffer B (50 mM potassium phosphate pH 6.0, 200 mM KCl and 5 mM β-mercaptoethanol) containing 3 M guanidinium hydrochloride and dialyzed extensively against buffer B without denaturant. This method produces SmpB protein that is >95% pure with a yield of 1–2 mg/l of cell culture. Purified SmpB is most soluble below pH 7, in the presence of reducing agents, and with NaCl or KCl at concentrations of 100 mM or more.

The N-terminal sequence of SmpB was determined by sequential Edman degradation by the MIT Biopolymers Facility. The CD spectrum of 10 μM SmpB was determined at 25°C using an AVIV 60DS spectrapolarimeter in buffer containing 50 mM potassium phosphate pH 6.0, 250 mM KCl, 5% glycerol and 1 mM DTT. Denaturation of SmpB protein by GuHCl was monitored by changes in CD ellipticity at 228 nm under the same temperature and buffer conditions but in the presence of increasing concentrations of denaturant.

Northern and Western blots

Cellular RNA was purified from log-phase cultures using the RNAeasy Total RNA Purification Kit (Qiagen), electrophoresed on 1.5% agarose and 20% formaldehyde gels, and transferred in 20× SSC to Nytron membranes (Schleicher and Schuell) using a Turboblot apparatus. Prehybridization and hybridization with appropriate ^{32}P-labeled DNA oligonucleotides complementary to SsrA RNA (5′-TAA AGC GTA GTT TTC GTC GTT TGC-3′) or 16S ribosomal RNA (5′-CCG TCC GCC ACT CGT CAG CAA-3′) were carried out according to Sambrook *et al*. (1989). To assay for correct processing of SsrA RNA, cellular RNA was electrophoresed on 4% polyacrylamide TBE gels in 8 M urea and detected by Northern blotting using the antisense SsrA probe (Li *et al*., 1998).

For Western blot analysis of λ-M2-H6-trpAt protein expression, cells were grown to mid-log phase in LB broth, induced with 1 mM IPTG, grown for varying times and lysed in SDS sample buffer. Total cellular proteins were electrophoresed on 15% acrylamide Tris–tricine gels and transferred to PVDF membranes. These blots were probed either with

anti-FLAG M2 antibodies (Eastman Kodak) or with antibodies that recognize the mutant AANDENYALDD tag (a gift of Eric Roche).

λ-M2-H6-trpAt purification and characterization

Cells were lysed in 50 mM Tris–HCl pH 8, 10 mM imidazole, 6 M GuHCl and λ-M2-H6-trpAt protein was purified by Ni-NTA chromatography (Qiagen) and dialyzed into 50 mM Tris–HCl pH 8, 50 mM KCl. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was performed using a Voyager-DE STR Biospectrometry Workstation (PerSeptive Biosystems).

Gel-mobility shift assay

A form of SsrA corresponding to the mature, processed RNA was transcribed *in vitro* using T7 polymerase in the presence of [α-^{32}P]UTP. Binding reaction mixtures (20 µl) contained 50 mM MES pH 6.5, 200 mM KCl, 5% glycerol, 5 mM β-mercaptoethanol, 0.01% NP-40, 0.1 mg/ml bovine serum albumin (BSA), labeled SsrA RNA (100 pM) and different amounts of SmpB protein. Mixtures were incubated at room temperature for 30 min and loaded onto a 5% acrylamide gel in TBE buffer (50 mM Tris pH 8.3, 25 mM borate and 2 mM EDTA). The gel was electrophoresed at 200 V, dried on gel-drying paper and exposed to BIOMAX MR film (Eastman Kodak) for 8–12 h at –80°C. Competition experiments were performed in the same manner except for inclusion of unlabeled SsrA or yeast tRNA (Sigma) in the reaction mixtures.

Fractionation and charging

Cell fractionation and ribosome association experiments were performed by the method of Komine *et al*. (1996). Mature SsrA RNA produced by transcription *in vitro* was charged with [^{3}H]alanine as described (Komine *et al*., 1994) using purified alanyl-tRNA synthetase (Ribas de Pouplana and Schimmel, 1997). His$_6$-tagged alanyl-tRNA synthetase was purified by Ni-NTA chromatography from *E.coli* strain TG1/pQE-875/pREP4 (a gift of Lluis Ribas de Pouplana and Paul Schimmel). SsrA RNA from wild-type and Δ*smpB* cells was purified as described (Komine *et al*., 1994).

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