

A tRNA-like structure is present in 10Sa RNA, a small stable RNA from *Escherichia coli*

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Communicated by Sidney Altman, June 13, 1994 (received for review May 6, 1994)

ABSTRACT We have determined that 10Sa RNA (one of the small stable RNAs found in *Escherichia coli*) has an interesting structural feature: the 5' end and the 3' end of 10Sa RNA can be arranged in a structure that is equivalent to a half-molecule (acceptor stem and TFC stem-loop) of alanine tRNA of *E. coli*. Primer-extension analysis of 10Sa RNA extracted from a bacterial mutant with temperature-sensitive RNase P function revealed that the precursor to 10Sa RNA (pre-10Sa RNA) is folded into a pre-tRNA-like structure *in vivo* such that it can be cleaved by RNase P to generate the 5' end of the mature 10Sa RNA. The purified 10Sa RNA can be charged with alanine *in vitro*. Disruption of the gene encoding 10Sa RNA (*ssrA*) caused a reduction in the rate of cell growth, which was especially apparent at 45°C, and a reduction in motility on semisolid agar. These phenotypic characteristics of the deletion strain (Δ *ssrA*) allowed us to investigate the effects of some mutations in 10Sa RNA *in vivo*, although the exact function of 10Sa RNA still remains unclear. When the G·U pair (G3·U357) in 10Sa RNA, which may be equivalent to the determinant G·U pair of alanine tRNA, was changed to a G·A or G·C pair, the ability to complement the phenotypic mutations of the Δ *ssrA* strain was lost. Furthermore, this inability to complement the mutant phenotypes that was caused by the substitution of the determinant bases by a G·A pair could be overcome by the introduction of a gene encoding alanyl-tRNA synthetase (*alaS*) on a multicopy plasmid. The evidence suggests that the proposed structural features of 10Sa RNA are indeed manifested *in vivo*.

10Sa RNA is one of the small stable RNAs that is found in *Escherichia coli*. It was first identified as 10S RNA, which was actually a mixture of 10Sa RNA and M1 RNA (originally designated 10Sb RNA) (1, 2). Subsequently, M1 RNA was shown to be a "catalytic RNA" (3), and it has been extensively studied from both a structural and a functional perspective (4). However, in contrast to M1 RNA, 10Sa RNA has not been the focus of much recent attention, and very little is known about either its structure or its function.

In our previous study, we found that the *ssrA* gene that encodes 10Sa RNA of *E. coli* is included in the genomes of phages 438, 439, and 440 of Kohara's library (5), which corresponds to 56.5 minutes on the linkage map of the chromosome (6). We cloned a 2.2-kb fragment that covers the overlapping region of these phages into a plasmid vector and determined the nucleotide sequence of the fragment.[§] We also purified 10Sa RNA from *E. coli* strain W3110 and determined its nucleotide sequence from both the 5' and the 3' end.

The nucleotide sequence of the *ssrA* gene from *E. coli* has already been reported by Chauhan and Apirion (7). In comparing our sequences with the published sequence, we no-

ticed an interesting structural feature of 10Sa RNA, as presented in Fig. 2—namely, 7 nucleotides at the 5' end and 28 nucleotides at the 3' end can be arranged in a structure that is equivalent to a half-molecule of tRNA. This half-molecule consists of an acceptor stem and a TFC stem-loop. In this paper, we describe the discovery of this unusual structural feature of 10Sa RNA and the results of several experiments that suggest that this structure actually exists *in vivo*.

MATERIALS AND METHODS

Bacterial and Phage Strains. *E. coli* K12 W3110 was used throughout this study. W3110 Δ *ssrA* is a deletion mutant in which the -10 promoter signal and about two-thirds of the coding region of *ssrA* have been deleted and replaced by a gene encoding the enzyme that confers kanamycin resistance. Strain ts709 is a temperature-sensitive mutant that is defective in the gene for RNase P (8). JC7623 *recB21 recC22 sbcB15 sbcC201* (9) was used for the disruption of the *ssrA* gene. Phage vector λ gtC (10) and phage P1 were from our laboratory stocks. Kohara's λ phage clone 439 (5) was used as a source of the *ssrA* gene for manipulation and for complementation tests with the Δ *ssrA* strain.

Media. The basic media used were Luria-Bertani (LB), LB containing an appropriate drug, and M9 minimal medium (11). For plating of λ phage, λ broth was used (12). Semisolid agar plates of LB medium plus 0.4% Bacto-agar (Difco) were used for examinations of bacterial motility.

DNA Clones. Plasmid vector Bluescript KS (-) was purchased from Toyobo (Osaka). p10SA is a plasmid clone of Bluescript KS (-) and carries the 2.2-kb *Pst* I-*Eco*RI fragment that contains the *ssrA* gene. p10SA-GA and p10SA-GC are derivatives of p10SA, in which the G·U pair in the terminal region of 10Sa RNA has been changed to a G·A pair and a G·C pair, respectively. pALAS is a plasmid clone carrying a 3.5-kb fragment that contains the entire gene encoding alanyl-tRNA synthetase (*alaS*) from pBR322. λ 10SA, λ 10SA-GA, and λ 10SA-GC are phage clones carrying the wild-type *ssrA* gene and mutant forms of this gene that were constructed with phage vector λ gt11.

Construction of the Strain with a Deletion of the *ssrA* Gene. A region including the *ssrA* gene in plasmid p10SA, between the *Acc* I restriction site at the site of the -10 promoter signal and the *Pvu* II site that is located two-thirds of the way along the coding region from the 5' end, was replaced by a gene conferring kanamycin resistance (Kan^R GenBlock in pUC4K; Pharmacia) by blunt-end ligation. Then, the *Pst* I-*Eco*RI insert of this plasmid was taken out and used to transform *E. coli* JC7623. In five of the kanamycin-resistant transformants obtained, the substitution was confirmed by Southern hybridization between the genomic DNA of each

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D12501).

transformant and a fragment of the *ssrA* gene. The disrupted *ssrA* gene in JC7623 cells was then transferred to W3110 cells by phage P1 using kanamycin resistance as a selectable marker. We confirmed that one of the transformants had the genetic markers of W3110 and failed to produce 10Sa RNA at a detectable level, and we designated this strain the W3110 Δ*ssrA* strain.

Purification of 10Sa RNA. An oligonucleotide probe, designated 10SA-1 (5'-TCGGCATGCACCTTGGGTTTCGCAA) and designed to hybridize with 10Sa RNA, was synthesized on a DNA synthesizer (model 380B; Applied Biosystems). Forty micrograms of this probe was biotinylated at the 3' end by use of biotin-21-dUTP (Clontech) and terminal deoxynucleotide transferase, and the DNA was then immobilized on 0.5 ml of streptavidin-conjugated agarose beads (ImmunoPure immobilized streptavidin; Pierce). The immobilized probe was allowed to hybridize with 5–10 mg of total RNA extracted from *E. coli* W3110 in 20 mM Pipes·NaOH (pH 6.4), 0.6 M NaCl, 0.2% SDS, 1 mM EDTA, and 50% (vol/vol) formamide, at 37°C. After a 16-hr incubation, the beads were washed twice with 2× SSC/0.2% SDS, twice with 0.2× SSC/0.2% SDS, and finally with H₂O at room temperature. Hybridized RNA was then eluted from the beads in a small volume of H₂O by incubation at 95°C for 2 min. 10Sa RNA was further purified by electrophoresis on a denaturing gel, since the sample eluted from the beads contained a small amount of rRNA.

Assays and Other Methods. Alanyl-tRNA synthetase was partially purified from an S100 extract of *E. coli* by ion-exchange chromatography (on DEAE-Toyopearl; Seikagaku Kogyo, Tokyo) as described by Andachi *et al.* (13). Fractions with the ability to aminoacylate tRNA^{Ala} were collected and concentrated (to a final concentration equivalent to an optical density at 280 nm of 9.6). The reaction mixture (50 μl) contained 50 pmol of 10Sa RNA or tRNA^{Ala} and 2.5 μl of the preparation of alanyl-tRNA synthetase, 30 μM L-[¹⁴C]alanine (6.0 GBq/mmol; Amersham), 100 mM Tris·HCl (pH 8.0), 10 mM KCl, 5 mM MgCl₂, 2 mM ATP, and 10 mM dithiothreitol. The mixture was incubated at 37°C and, at the indicated times, 9.5-μl aliquots were removed and spotted on disks of filter paper. The disks were washed with ice-cold 5% (wt/vol) trichloroacetic acid and ethanol, dried, and monitored for radioactivity in a liquid scintillation counter.

The synthetic oligonucleotides 10SA-1 (5'-TCGGCATGCACCTTGGGTTTCGCAA), for primer extension analysis, and 10SA-mix (5'-TTGGTGGT/GGCTGGCGGG), for *in vitro* mutagenesis to alter the terminal region of 10Sa RNA, were synthesized with the DNA synthesizer. Primer extension was performed as described by Boorstein and Craig (14) using avian myeloblastosis virus reverse transcriptase XL (Takara Shuzo, Kyoto). *In vitro* mutagenesis was performed using a Muta-Gene kit (Bio-Rad).

The sequence of 10Sa RNA was determined with an RNA sequencing system (Bethesda Research Laboratories). For DNA sequencing of the *ssrA* gene, deletion mutants of p10SA in pUC118 and pUC119 were obtained as described by Yanisch-Perron *et al.* (15) and sequenced by the dideoxy chain-termination method (16).

RESULTS

An Unusual Structural Feature of 10Sa RNA. As a result of DNA and RNA sequencing, we confirmed the primary structure of the *ssrA* gene (Fig. 1). The gene is 363 bp long, and it has its own promoter and a *p*-independent terminator, forming a single-gene operon. Looking at its nucleotides sequence, we realized that 7 nucleotides at the 5' end and 28 nucleotides at the 3' end could be arranged in a structure that is equivalent to a half-molecule of tRNA and consists of an acceptor stem and a TFC stem-loop (Fig. 2). All of the

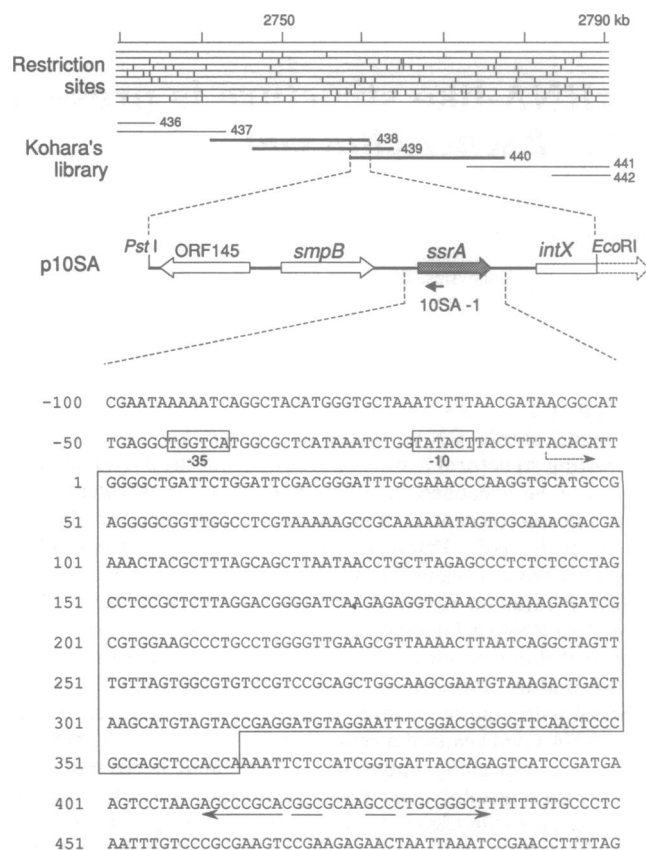


FIG. 1. The location and the nucleotide sequence of the *ssrA* gene. The restriction map at the top is redrawn from Kohara *et al.* (5). The sites indicated are those recognized by *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV, *Bgl* I, *Kpn* I, *Pst* I, *Pvu* II (from the top line to the bottom line). The entire sequence of p10SA can be found in the GenBank data base (accession no. D12501). The indicated genes other than *ssrA* are ORF145, an unidentified open reading frame encoding 145 amino acids; *smpB*, a gene that encodes a small protein with unknown function (17); and *intX*, a truncated open reading frame that encodes an integrase-like protein (Y.K. and H.I., unpublished results). In the sequence of *ssrA*, the coding region and the consensus sequences of the promoter (-10 and -35) are boxed, the inverted repeat of the terminator is indicated by arrows, and the site of initiation of transcription is indicated by a bent arrow. 10SA-1 is a synthetic primer used in primer-extension analysis (see legend to Fig. 3).

conserved bases known to occur in these domains of standard tRNAs, including the 3'-CCA sequence, can be found at the corresponding positions in 10Sa RNA.

Chauhan and Apirion (7) reported the nucleotide sequence of the *ssrA* gene from *E. coli*. Although their DNA sequence is identical to ours, with the exception of a few bases, the 5' and 3' ends of the coding region that they determined differ from those of our sequence. Differences between the sequence in Fig. 1 and that of Chauhan and Apirion (7) are as follows: G340 and G411 in Fig. 1 are missing, and another G is added after G333 in the sequence in ref. 7; AGCCC (425–429) in our sequence is replaced by CCAG in the sequence in ref. 7; and the coding region for 10Sa RNA in ref. 7 extends from A (-5) to T357 in Fig. 1.

Brown *et al.* (18) and Tyagi and Kinger (19) reported the nucleotide sequences of the genes for 10Sa RNAs from *A. eutrophus* and *M. tuberculosis*, respectively. Both groups defined the coding regions in accordance with the report of Chauhan and Apirion (7). If these coding regions are defined by reference to our data, the 10Sa RNAs from both bacteria can be drawn with tRNA-like structures similar to that of 10Sa RNA from *E. coli*. The structures include a G-U pair at

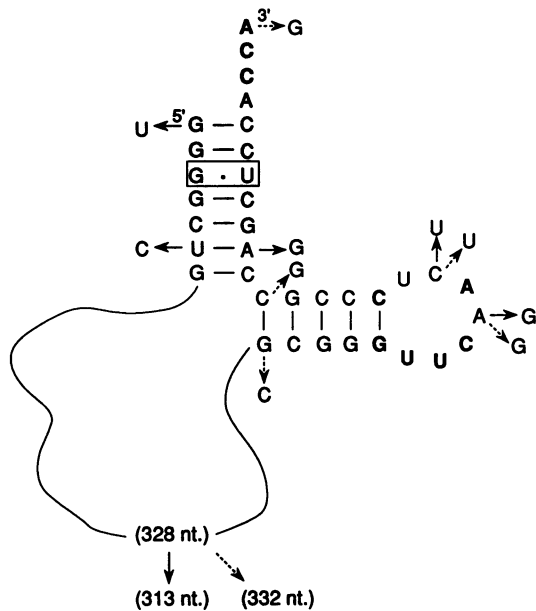


FIG. 2. The tRNA-like structure of 10Sa RNA. Seven nucleotides from the 5' end and 28 nucleotides from the 3' end of 10Sa RNA can be arranged in a structure equivalent to a half-molecule of tRNA. This structure consists of an acceptor stem and a TFC stem-loop. The conserved bases found in these domains of normal tRNAs are indicated in boldfaced letters. The nucleotide substitutions found in 10Sa RNA from *Alcaligenes eutrophus* and *Mycobacterium tuberculosis* are indicated with solid arrows and broken arrows, respectively.

the third position of the acceptor-like stem, although U at the 5' end of the 10Sa RNA from *A. eutrophus* cannot form a Watson-Crick base pair (Fig. 2).

The 5' End of Mature 10Sa RNA Is Generated by RNase P. In *E. coli* (and in other organisms), tRNA precursors are cleaved by RNase P to generate the 5' ends of the mature tRNAs. It was reported previously that the smallest substrate derived from a pre-tRNA that can be cleaved efficiently by RNase P (or by M1 RNA) retained only the domains of the aminoacyl stem and the TFC stem-loop (20). By primer-extension analysis, using a mutant with temperature-sensitive RNase P function, we observed that a precursor for 10Sa RNA (pre-10Sa RNA), which contains an extra 7 nucleotides at the 5' end, accumulates when RNase P is inactivated (Fig. 3). This observation suggests that pre-10Sa RNA is folded into a pre-tRNA-like structure *in vivo* and is cleaved by RNase P to generate the 5' end of mature 10Sa RNA.

Aminoacylation of 10Sa RNA with Alanine *in Vitro*. 10Sa RNA has a G-U base pair (G3·U357) in its tRNA-like structure that corresponds to the determinant G-U pair (G3·U70) in the acceptor stem of tRNA^{Ala} (21, 22). Furthermore, it is known that a minihelix, which consists of the acceptor stem and TFC stem-loop, can be a substrate for alanyl-tRNA synthetase, provided that there is a G-U pair at the third position of the stem (23). Therefore, we examined whether 10Sa RNA could serve as a substrate for alanyl-tRNA synthetase *in vitro*. The results shown in Fig. 4 indicate that purified 10Sa RNA can indeed be charged with alanine by partially purified alanyl-tRNA synthetase from *E. coli*. In this experiment, only about half of the 10Sa RNA molecules were charged with alanine, and this level varied among preparations of 10Sa RNA. We suspect that this phenomenon may have been due to some conformational variability by unmodified nucleotides or due to contamination of the preparation of 10Sa RNA by molecules of 10Sa RNA that lacked a few bases at their ends and could not be separated from the

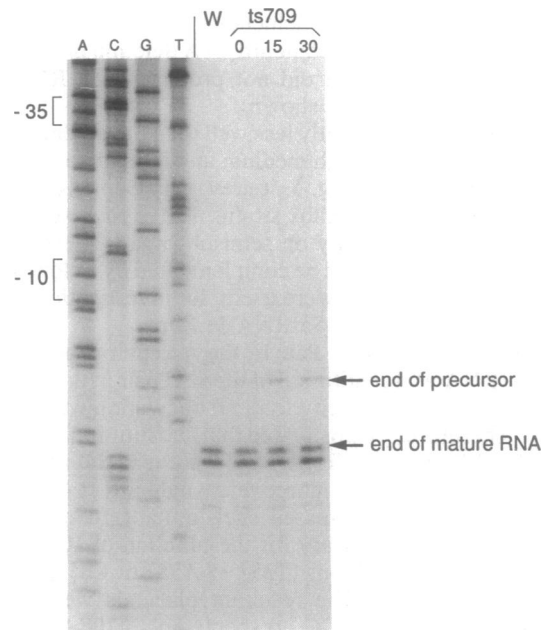


FIG. 3. Processing of the 5' end of the 10Sa RNA *in vivo*. ts709, a mutant with temperature-sensitive RNase P function (8), was grown until early logarithmic phase at 32°C; then the culture was shifted to 42°C. Total RNA was extracted from aliquots of the culture at 0, 15, and 30 min after the shift to 42°C (lanes ts709: 0, 15, 30), as it was from a standard culture of the wild-type strain W3110 grown at 37°C (lane W). Primer-extension analysis was performed using these RNA samples and the synthetic oligonucleotide 10SA-1 (5'-TCGGCATGCACCTGGGTTTCGCAA) by the methods described by Boorstein and Craig (14). The four lanes on the left represent the results of dideoxynucleotide sequencing reactions with 10SA-1 as a primer, and the positions of the promoter sequences (-10, -35) are indicated.

complete molecules. We did not examine the possibility that 10Sa RNA might also be charged with other amino acids since 10Sa RNA does not contain any of the determinant bases of other tRNAs identified to date.

Construction of a Strain with a Deletion of the *ssrA* Gene. We constructed a strain in which the gene conferring kanamycin resistance replaced part of the *ssrA* gene (from the -10 promoter signal to a site about two-thirds of the way along the coding region) from strain JC7623. The disrupted *ssrA* gene was then transferred to W3110 cells by phage P1 using

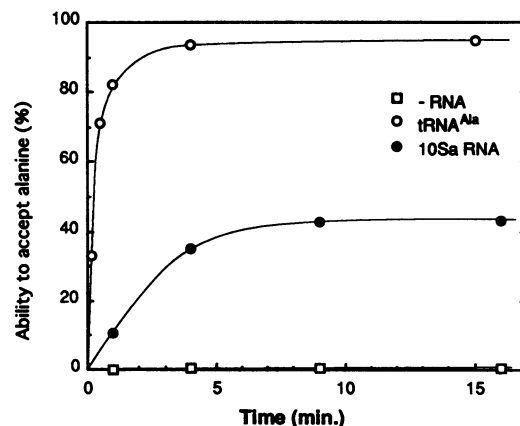


FIG. 4. Aminoacylation of 10Sa RNA *in vitro*. Alanyl-tRNA synthetase was partially purified from an S100 extract of *E. coli*. The assay conditions are described in *Materials and Methods*. 10Sa RNA was purified from W3110 that carries p10SA, and purified tRNA^{Ala} from *E. coli* was purchased from Subriden RNA.

kanamycin resistance as a selectable marker. We used this strain (W3110 Δ ssrA) as a standard ssrA deletion mutant after we had confirmed that it did not produce 10Sa RNA at a detectable level (data not shown).

W3110 Δ ssrA grew slightly less well than the parental strain at 37°C and at 42°C, in rich medium and in minimal medium. However, as shown in Fig. 5, it grew very poorly at 45°C as compared with the parental strain. The Δ ssrA strain also exhibited reduced motility on semisolid agar. These phenotypic characteristics could be complemented by a cloned *ssrA* gene (Fig. 5). Thus, they were useful for investigations of the effects of mutations in 10Sa RNA *in vivo*.

Substitution of the G·U Pair in the Acceptor Stem of 10Sa RNA. In tRNA^{Ala}, substitution of the G·U pair in the acceptor stem eliminates the capacity for aminoacylation with alanine (21, 24). Therefore, we postulated that substitution of the G·U pair (G3·U357) in 10Sa RNA would prevent the aminoacylation of 10Sa RNA and, moreover, would affect the phenotype of strains that expressed such mutant 10Sa RNAs if aminoacylation is necessary for the function of 10Sa RNA.

We introduced mutations (T357 \rightarrow A357 or C357) into the *ssrA* gene of p10SA. The resultant plasmids were named p10SA-GA and p10SA-GC, respectively. p10SA-GA was able to complement one of the mutant phenotypes of the Δ ssrA strain—namely, the reduced motility on semi-solid agar—as could the wild-type plasmid p10SA. However, p10SA-GC failed to complement this phenotype and, furthermore, it caused a reduction in the growth rate of the Δ ssrA strain under normal growth conditions (Fig. 6A). We could not examine the effects of these mutant plasmids on the other phenotype—namely, temperature sensitivity at 45°C—since cells were unable to retain high-copy-number plasmids, such as Bluescript, at 45°C. The mutant *ssrA* genes were then cloned into a λ phage vector, and the recombinant phages (λ 10SA-GA and λ 10SA-GC) were used to lysogenize the Δ ssrA strain so that we could examine the effects of the mutant genes as single copies. As shown in Fig. 6B, neither λ 10SA-GA nor λ 10SA-GC complemented the two mutant phenotypes of Δ ssrA, temperature sensitivity at 45°C and reduced motility on semisolid agar. These results indicate that a mutant 10Sa RNA with G·C pair instead of the G·U pair hardly functions at all. By contrast, another mutant 10Sa RNA with a G·A pair instead of a G·U pair is partially functional *in vivo*.

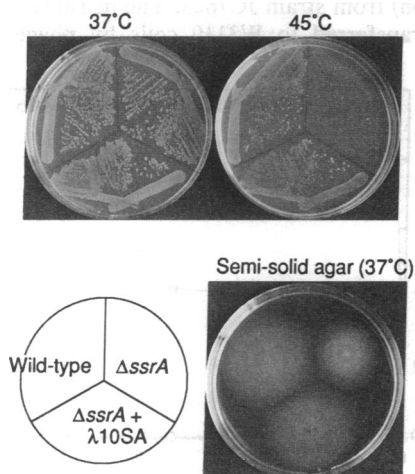


FIG. 5. Phenotypic characteristics of the Δ ssrA strain. The wild-type strain, the Δ ssrA strain, and the Δ ssrA strain lysogenized with λ 10SA and wild-type λ (in order to suppress the *cI₈₅₇* mutation of the vector phage λ gt11) were spread on LB plates and incubated at 37°C and 45°C, or cells were spotted on a semisolid agar plate and incubated at 37°C for an examination of cell motility.

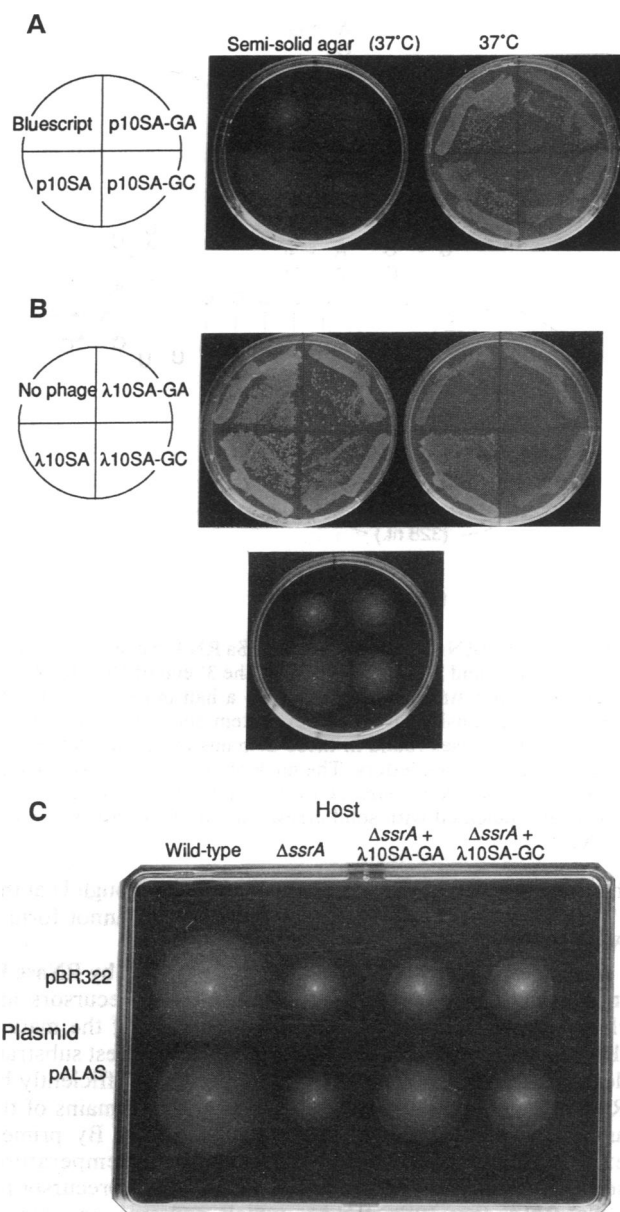


FIG. 6. Functional analysis of mutant 10Sa RNAs *in vivo*. (A) Δ ssrA strains carrying p10SA, p10SA-GA, p10SA-GC, and Bluescript KS(-) were examined for cell motility and colony growth at 37°C. (B) The Δ ssrA strain and the same strain lysogenized with λ 10SA, λ 10SA-GA, and λ 10SA-GC (together with wild-type λ) were examined for motility and temperature sensitivity at 45°C. (C) pALAS (including the *alaS* gene) and pBR322 were introduced into the wild-type strain, the Δ ssrA strain, and the two lysogens mentioned in B (λ 10SA-GA and λ 10SA-GC), and the motility of cells was examined on a semisolid agar plate.

We prepared transcripts *in vitro* with the same nucleotide sequences as those of 10Sa RNA and two mutants (with A357 or C357) by the method of Sampson and Uhlenbeck (25), and we measured their abilities to accept alanine *in vitro*. The wild-type transcript could be charged with alanine to a significant level, albeit to a lower level than native 10Sa RNA. Neither the C357 nor the A357 mutant transcript could accept alanine at more than a barely detectable level (about 3% of the accept or capacity of the wild-type transcript; data not shown). These results showed that replacement of the G·U pair in 10Sa RNA by another pair of nucleotides prevents its aminoacylation, at least to some extent, as is the case for tRNA^{Ala} described by McClain and Foss (22) (i.e., the third

position G-C mutant of tRNA^{Ala} was weakly active with a 60-fold reduced efficiency of suppression, whereas the G-A mutant was partially active with the 6-fold reduced efficiency).

Effects of Overexpression of Alanyl-tRNA Synthetase on the Phenotype of Cells with Mutant *ssrA* Genes. To confirm the involvement of aminoacylation in the function of 10Sa RNA, we examined the effects of overexpression of alanyl-tRNA synthetase on the phenotype of cells with mutant *ssrA* genes. We cloned a 3.5-kb fragment that contained the entire *alaS* gene (which encodes alanyl-tRNA synthetase) into plasmid pBR322 to generate pALAS. pALAS and pBR322 were introduced into the lysogens described above. Only the λ 10SA-GA lysogen was complemented by the multicopy *alaS* gene, as judged by the enhanced motility of cells on semisolid agar (Fig. 6C). Although the complementation by *alaS* was incomplete, the result was reproducible. This experiment suggests that the mutant 10Sa RNA with the G3-A357 pair has a reduced ability to accept alanine, even though this ability was too low to detect in our charging experiment *in vitro*. From these results, we conclude that aminoacylation of the 10Sa RNA might be associated with its function, although at present we cannot exclude a possibility that only the binding of the 10Sa RNA with alanyl-tRNA synthetase is related to the function.

DISCUSSION

10Sa RNA has been found in many species of microorganisms—for example, *A. eutrophus* (18), *M. tuberculosis* (19), *Bacillus subtilis*, and *Mycoplasma* (A. Muto, personal communication). In all cases, the 10Sa RNA can be drawn with a tRNA-like structure that resembles that proposed for the 10Sa RNA from *E. coli*. This observation strengthens the argument that the tRNA-like structure presented herein is real and suggests that this small stable RNA might play an important role in bacterial cells.

Recently, Retallack *et al.* (26) reported that the lack of functional 10Sa RNA appears to be responsible for the inhibition of growth of λ -P22 hybrid phage. They proposed that 10Sa RNA acts directly or indirectly to facilitate removal of C1 protein from its target site on the DNA. At this time, we have no idea about the relationship between the proposed tRNA-like structure of 10Sa RNA and the evidence related to the hybrid phage growth presented by Retallack *et al.* (26).

Oh and Apirion (27) reported that disruption of the *ssrA* gene had a significant effect on cell growth at all temperatures. Both our Δ *ssrA* strains, W3110 Δ *ssrA* and JC7623 Δ *ssrA*, the latter having the same background as their mutant, grew slightly more slowly than the parental strain at 37°C and at 42°C, but the growth defect was smaller than that reported by Oh and Apirion (27). The main difference between their mutant and ours is as follows. In their mutant, the promoter of the *ssrA* gene is intact and the interrupted gene is still transcribed. By contrast, almost the entire gene, including the -10 promoter signal, is absent and no RNA related to 10Sa RNA is expressed in our strains. We can assume, therefore, that the interrupted 10Sa RNA might have had a harmful effect in their mutant strain.

We cannot explain why disruption of the *ssrA* gene reduces the rate of cell growth at 45°C. We are also unable at present to offer a definitive explanation for the reduced motility of the Δ *ssrA* strain. Many factors affect the motility of cells on semisolid agar (28, 29), and this issue remains to be resolved.

Recently, in our laboratory, we isolated a temperature-sensitive mutant from the Δ *ssrA* strain. The strain is unable to grow at 42°C but it does grow at 42°C upon introduction of a wild-type *ssrA* gene, but not a mutant *ssrA* gene such as that with A357 or C357, via phage or plasmid. This temperature-

sensitive mutation is also complemented by lysogenization with Kohara's phage clone 246 or 247, which are derived from a region at 27 minutes on the linkage map of *E. coli* (H. Ando, M.K., and H.I., unpublished results). The product encoded by the gene carried by both of the phage clones could somehow associate with 10Sa RNA in a functional manner. Thus, we expect that identification of the relevant gene in the new temperature-sensitive mutant might be useful for elucidation of the function of 10Sa RNA.

In this report, we have demonstrated that 10Sa RNA of *E. coli* includes a structure equivalent to a half-molecule of tRNA and that the aminoacylation of 10Sa RNA plays a role in the function of this small stable RNA. Further genetic and biochemical studies of the function of 10Sa RNA, including the role of aminoacylation, should reveal new and interesting features of this molecule.

This research was supported by a Grant-in-Aid for Scientific Research on Priority Areas (no. 04272104) from the Ministry of Education, Science, and Culture of Japan.

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