RNA (2001), *7*:1708–1716. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838201011281

REPORT

Increased sensitivity to protein synthesis inhibitors in cells lacking tmRNA

JESÚS DE LA CRUZ[1,2] and AGUSTÍN VIOQUE[1]

[1]Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Américo Vespucio s/n, E-41092 Sevilla, Spain

ABSTRACT

tmRNA (also known as SsrA or 10Sa RNA) is involved in a *trans*-translation reaction that contributes to the recycling of stalled ribosomes at the 3′ end of an mRNA lacking a stop codon or at an internal mRNA cluster of rare codons. Inactivation of the *ssrA* gene in most bacteria results in viable cells bearing subtle phenotypes, such as temperature-sensitive growth. Herein, we report on the functional characterization of the *ssrA* gene in the cyanobacterium *Synechocystis* sp. strain PCC6803. Deletion of the *ssrA* gene in *Synechocystis* resulted in viable cells with a growth rate identical to wild-type cells. However, null *ssrA* cells (Δ*ssrA*) were not viable in the presence of the protein synthesis inhibitors chloramphenicol, lincomycin, spiramycin, tylosin, erythromycin, and spectinomycin at low doses that do not significantly affect the growth of wild-type cells. Sensitivity of Δ*ssrA* cells similar to wild-type cells was observed with kasugamycin, fusidic acid, thiostrepton, and puromycin. Antibiotics unrelated to protein synthesis, such as ampicillin or rifampicin, had no differential effect on the Δ*ssrA* strain. Furthermore, deletion of the *ssrA* gene is sufficient to impair global protein synthesis when chloramphenicol is added at sublethal concentrations for the wild-type strain. These results indicate that ribosomes stalled by some protein synthesis inhibitors can be recycled by tmRNA. In addition, this suggests that the first elongation cycle with tmRNA, which incorporates a noncoded alanine on the growing peptide chain, may have mechanistic differences with the normal elongation cycles that bypasses the block produced by these specific antibiotics. tmRNA inactivation could be an useful therapeutic target to increase the sensitivity of pathogenic bacteria against antibiotics.

Keywords: antibiotics; *ssrA*; *Synechocystis*; tmRNA; translation

INTRODUCTION

Bacterial cells contain a panoply of stable nontranslated RNAs such as ribosomal and transfer RNAs (rRNAs and tRNAs; reviewed in Wassarman et al., 1999). Amongst these RNAs, the so-called tmRNA (also known as SsrA or 10Sa RNA) was one of the first to be identified in *Escherichia coli* (Jain et al., 1982); however, its function has remained elusive for a decade The tmRNA is encoded by the *ssrA* gene, which is highly conserved in all eubacteria (Knudsen et al., 2001). In vivo, tmRNA is tightly associated to the SmpB protein and less tightly to other proteins (Karzai et al., 1999; Karzai & Sauer, 2001). Different evidence indicates that tmRNA plays a role in translation (for a review, see Muto et al., 1998; Karzai et al., 2000). tmRNA can form in vivo a tRNA half molecule consisting of a amino acid acceptor arm and a TΨC stem-loop (Komine et al., 1994). The presence of this tRNA-like structure is further supported by the fact that the precursor of tmRNA (pre-tmRNA), as all pre-tRNAs, is 5′-end processed by RNase P, and tmRNA is aminoacylated by alanyl-tRNA synthetase (Komine et al., 1994). In addition, the alanylated-tmRNA interacts with elongation factor Tu (EF-Tu) and GTP forming a ternary complex (Rudinger-Thirion et al., 1999) and is found associated with 70S ribosomes (Tadaki et al., 1996). Finally, tmRNAs have a short reading frame coding for about 11 amino acids (Knudsen et al., 2001), which are added to the C-terminus of an incomplete nascent protein translated from truncated mRNAs (Keiler et al., 1996).

Based on some of these observations, Sauer and coworkers proposed the "*trans*-translation" model for the biological function of tmRNA (Keiler et al., 1996).

Reprint requests to: Agustín Vioque, Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-CSIC, Centro de Investigaciones Científicas Isla de la Cartuja, Avda. Américo Vespucio s/n, E-41092 Sevilla, Spain; e-mail: vioque@us.es.

[2]Present address: Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Reina Mercedes 6, E-41012 Sevilla, Spain.

According to this model, alanine-charged tmRNA associated with EF-Tu enters the A site of a ribosome stalled at the 3′ end of a truncated mRNA lacking a stop codon. Then, the noncoded alanine is incorporated to the nascent polypeptide chain through a cycle of translation elongation, before the mRNA-like domain of tmRNA replaces the truncated mRNA on the ribosome. Then, this domain is used as a template to add a short peptide to the nascent polypeptide before translation terminates and a tagged protein is released (for details, see Keiler et al., 1996; Muto et al., 1998). The short peptide acts as a recognition sequence that directs the protein to quick degradation by specific proteases (Gottesman et al., 1998; Levchenko et al., 2000). Therefore, tmRNA has a double function: First, ribosomes that are stalled at truncated mRNAs lacking a termination codon are released. Second, the prematurely truncated proteins are rapidly degraded, avoiding accumulation. Both function have been separated genetically by engineering cells that carry a *ssrA* gene coding for an altered tag that is resistant to proteases, or alternatively, that cannot be aminoacylated (Keiler et al., 1996; Withey & Friedman, 1999; Huang et al., 2000).

From these studies, it seems clear that ribosome release and not protein tagging is the relevant function of tmRNA (Keiler et al., 1996; Withey & Friedman, 1999; Huang et al., 2000). In addition, it has been demonstrated that tmRNA-mediated *trans*-translation occurs quite frequently in normally growing cells and not only at truncated mRNAs lacking a termination codon (Abo et al., 2000). Tagging by tmRNA occurs at rare codons in an mRNA or even at the natural termination codon (Roche & Sauer, 1999, 2001). This suggests that ribosome pausing or stalling by a number of different causes can activate tmRNA function. However, several questions remain unsolved: How does tmRNA recognize stalled ribosomes, what is the mechanism of incorporation of the noncoded alanine, and how does the ribosome resume in-frame translation at the mRNA-like domain of tmRNA?

Moreover, despite its important biological function and its ubiquitous presence in the eubacterial kingdom, the precise physiological role of tmRNA still remains elusive,as it is, in general, dispensable for cell viability and it has been proved to be essential only in *Neisseria gonorrhoeae* and *Mycoplasma* (Hutchison et al., 1999; Huang et al., 2000). However, cells lacking a functional tmRNA exhibit a variety of subtle phenotypes such as slow growth at high temperature or under different stresses, reduced motility, inhibition of phage growth, an increase in the activity of an unidentified protease, enhanced activity of several repressors or reduced pathogenesis (Karzai et al., 2000; Muto et al., 2000; and references therein). Interestingly, Sugita and coworkers could not inactivate the *ssrA* gene in the cyanobacterium *Synechococcus* sp. strain PCC6301, suggesting an essential function for this gene (Watanabe et al., 1998). For this reason, we undertook the functional characterization of the *ssrA* gene in the cyanobacterium *Synechocystis* sp. strain PCC6803, in the context of an ongoing project on the analysis of stable RNAs from this cyanobacterium. Herein, we report that *Synechocystis* cells disrupted for the *ssrA* gene (Δ*ssrA* cells) show no growth defect phenotype under normal laboratory conditions. However, a Δ*ssrA* strain is not viable when cultivated at low concentrations of several protein synthesis inhibitors. In addition, global protein synthesis is impaired at low concentration of chloramphenicol in a Δ*ssrA* strain but not in a wild-type strain. We conclude that tmRNA has a role in recycling stalled ribosomes when they are blocked by those protein synthesis inhibitors. We assume that the first elongation cycle that incorporates the noncoded alanine to nascent polypeptide chains is resistant to the bound antibiotic.

RESULTS AND DISCUSSION

tmRNA is dispensable for growth

To study the cellular function of *Synechocystis* tmRNA, we first disrupted the *ssrA* gene by replacing most of the gene sequence with a kanamycin resistance (Km^r) cassette (Fig. 1A). Although the *ssrA* gene is not part of a polycistronic unit (Kaneko et al., 1996), the cassette was inserted in both orientations to control for hypothetical polar effects. The disrupted copy of *ssrA* could be completely segregated as tested by southern blot (data not shown). Therefore, cells lacking a functional *ssrA* gene seem to be viable.

To further confirm the nonessential role of tmRNA, we determined its steady-state levels by northern blot and primer extension analyses in wild-type and Δ*ssrA* strains. As seen in Figure 1B,C, disruption of the *ssrA* gene resulted in the loss of mature tmRNA. In addition, we detected an additional band in the wild-type strain by primer extension analysis (Fig. 1C, lane 1), which we attribute to the precursor of tmRNA (Tous et al., 2001). Altogether, these experiments indicate that *ssrA* is a dispensable gene in *Synechocystis*, in disagreement with the data from Sugita and coworkers for *Synechococcus* (Watanabe et al., 1998).

Disruption of *ssrA* causes no apparent growth defect

To understand the nonessential role of tmRNA, the growth rate of the Δ*ssrA* strain was compared to that of the wild-type strain. As seen in Figure 2A, when cells were cultivated in nitrate containing BG-11 medium at 30 °C (see Materials and Methods), the growth rates were identical for wild-type and Δ*ssrA* strains. In addition, Δ*ssrA* cells were as motile as wild-type cells and we could not detect any morphological difference when

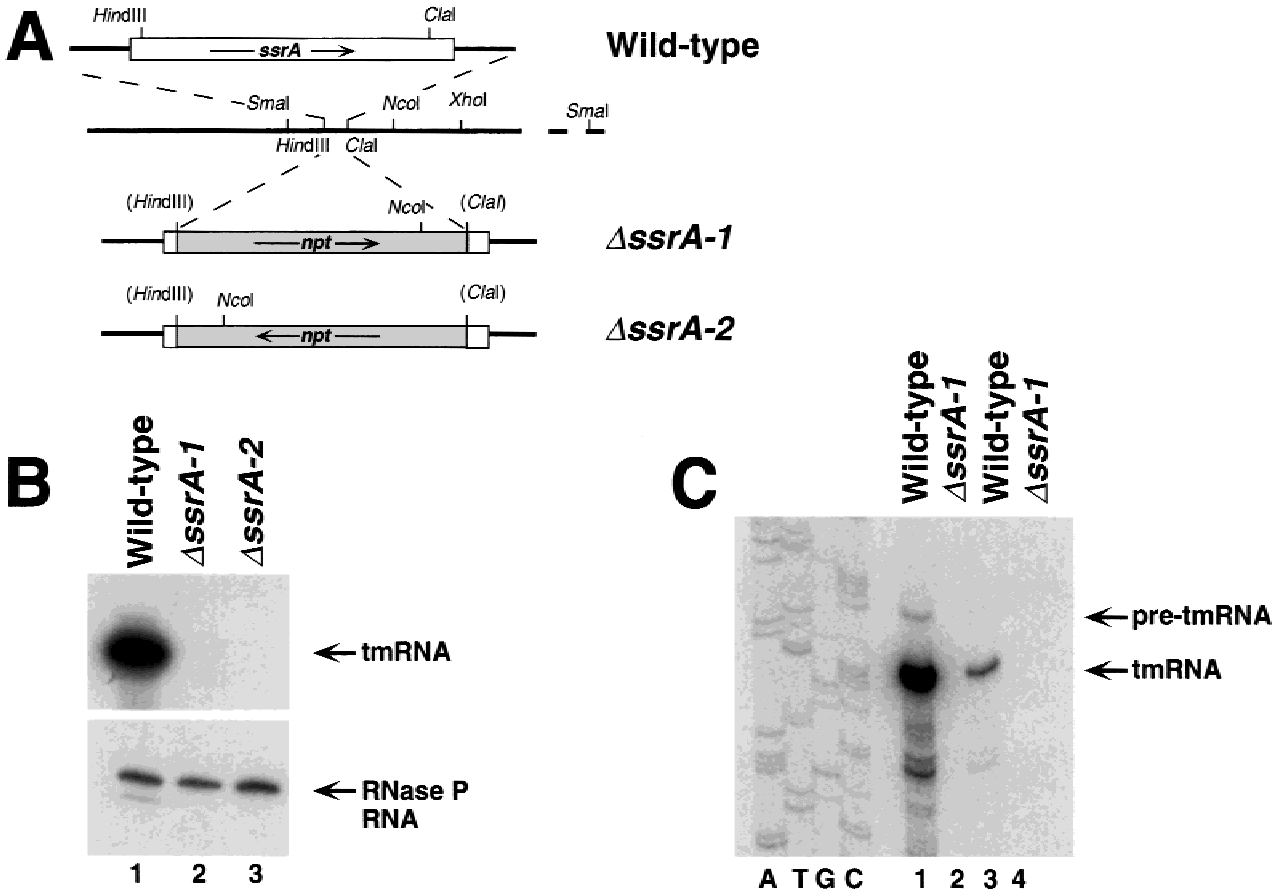

FIGURE 1. Disruption of the *Synechocystis ssrA* gene. **A:** Schematic representation of the *Synechocystis* genomic region where the *ssrA* gene is located. A *Hin*cII Km^r cassette (1.3 kb) containing the neomycin phosphotransferase gene (*npt*) was inserted between the blunt-ended *Hin*dIII and *Cla*I sites in both orientations (Δ*ssrA-1* and Δ*ssrA-2*). **B:** Northern blot analysis; total RNA was extracted from wild-type and null *ssrA* (Δ*ssrA-1* and Δ*ssrA-2*) cells and hybridyzed with a *Sma*I-*Nco*I fragment containing the *ssrA* gene (upper panel) or a *rnpB* gene probe (bottom panel; Vioque, 1992). **C:** Primer extension using the oligonucleotide 10SAPE (see Materials and Methods) on total RNA extracted from wild-type and null *ssrA* (Δ*ssrA-1* and Δ*ssrA-2*) cells. Arrows points to the extension products identifying the putative transcription start site (pre-tmRNA) and the 5′ end of mature tmRNA (tmRNA) only in wild-type cells. For the primer extension shown in lanes 3 and 4, a 10-fold molar excess of cold primer was mixed with the radiolabeled primer before annealing. A sequencing reaction on the wild-type *ssrA* gene using the same primer is also shown.

cells were observed under the light microscope (data not shown). We conclude that *Synechocystis* tmRNA is totally dispensable under normal laboratory growth conditions.

tmRNA requirement under several stresses: Sensitivity to protein synthesis inhibitors

To estimate differences in growth rates under different stressful conditions, serial dilutions of cultures from wild-type and Δ*ssrA* cells were spotted on BG11 medium plates, and plates were incubated at 30 °C (see Materials and Methods). Stressful conditions were: exposure to UV irradiation (2,000 KJ·s^{-1}) for 2 to 10 min, DTT (250 mM), 2-mercaptoethanol (5 mM), treatment at 25 °C, 37 °C, and 45 °C for 30 min. In all conditions, no differences in the survival or the growth rates of the wild-type and Δ*ssrA* strains were found (data not shown).

Because tmRNA plays a role on translation (see Introduction), we also measured growth rates of both strains in the presence of low concentrations of antibiotics affecting protein synthesis. As seen in Table 1 and Figure 2B, the Δ*ssrA* strains do not grow in the presence of sublethal concentrations for the wild-type strain of chloramphenicol, erythromycin, lincomycin, spectinomycin, spiramycin, and tylosin. These data indicate that the survival rate of the wild-type strain is 10- to 1000-fold higher than that of the Δ*ssrA* strains (Fig. 2B). Interestingly, all these drugs except spectinomycin have been described to bind to the peptidyl transferase center, which has been localized within domain V of the 23S rRNA (Gale et al., 1981; Rodríguez-Fonseca et al., 1995; Nissen et al., 2000; Poulsen et al., 2000). Furthermore, all these drugs except erythromycin and spectinomycin inhibit the peptidyl transferase reaction (Gale et al., 1981). Re-

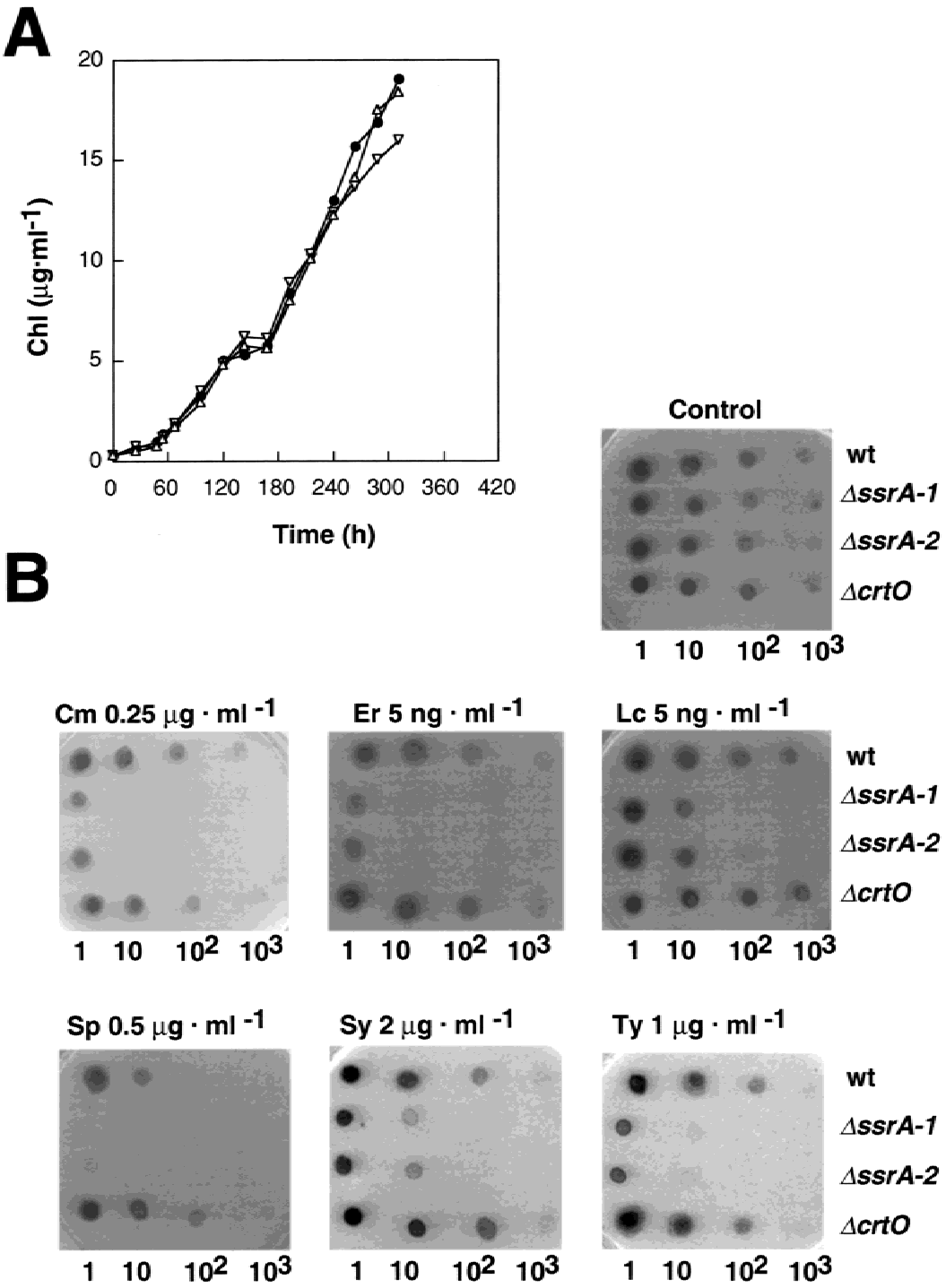

FIGURE 2. tmRNA is required for survival in the presence of protein synthesis inhibitors. **A:** Growth comparison of wild-type and Δ*ssrA* strains. Cultures were inoculated at an initial concentration of 0.5 $\mu g \cdot mL^{-1}$ chlorophyll. Cells were grown on nitrate containing BG11 medium for up to 12 days and growth rate was measured as the concentration of chlorophyll per milliliter of culture. Wild-type (●), Δ*ssrA-1* (△) and Δ*ssrA-2* (▽) strains. **B:** Cells from wild-type (wt), null *ssrA* (Δ*ssrA-1* and Δ*ssrA-2*) and null *crtO* (Δ*crtO*) strains were grown to 3 OD_{750} units. Serial dilutions (dilution factor 1 to 10^3) were spotted onto nitrate containing BG11 medium plates at the indicated concentrations of antibiotics. Plates were incubated as described in Materials and Methods for 7 days. Control: no antibiotic added; Cm: chloramphenicol; Er: erythromycin; Lc: lincomycin; Sp: spectinomycin; Sy: spiramycin; Ty: tylosin.

cently, it has been shown that erythromycin binds to the polypeptide tunnel, suggesting a simple steric-hindrance mechanism of inhibition of elongation (Gabashvili et al., 2001). Spectinomycin also inhibits translocation, but it binds to specific sites on 16S rRNA (Bilgin et al., 1990; Carter et al., 2000).

TABLE 1. Effect of different antibiotics on the growth rate of wild-type and Δ*ssrA* cells from *Synechocystis* sp. PCC6803.

Inhibitor	Function affected	Growth difference[a]
Ampicillin	Cell wall synthesis	None
Rifampicin	Transcription	None
Kasugamycin	Initiation (fMet-tRNA binding)	None
Kanamycin	Translation accuracy	+
Neomycin	Translation accuracy	+
Streptomycin	Translation accuracy	+
Tetracycline	aa-tRNA·EF-Tu·GTP binding	None
Chloramphenicol	Peptide bond formation	−
Lincomycin	Peptide bond formation	−
Spiramycin	Peptide bond formation	−
Tylosin	Peptide bond formation	−
Erythromycin	Elongation	−
Spectinomycin	Translocation (EF-G·GTP interaction)	−
Fusidic acid	Translocation (EF-G·GDP release)	None
Thiostrepton	Translocation (EF-G·GDP turnover)	None
Puromycin	Elongation	None

Relative growth rate was compared by dotting serial dilutions of exponentially growing cells on plates containing the indicated antibiotics at sublethal concentrations for the wild-type strain (see Materials and Methods).

[a]None: similar growth of wild-type and Δ*ssrA* strains; +: better growth of the Δ*ssrA* strain; −: better growth of the wild-type strain. Note that the better growth of the Δ*ssrA* strains in kanamycin, neomycin, and streptomycin is not related to the loss of tmRNA function but to the introduced Km^r cassette following transformation.

As also shown in Table 1, antibiotics affecting translation initiation or EF-Tu and EF-G-mediated steps during translocation (except erythromycin and spectinomycin) do not differentially affect the Δ*ssrA* strain as compared to the wild-type strain. In addition, the Δ*ssrA* strains grow better than the wild-type strain in the presence of any concentration of kanamycin, neomycin, or streptomycin. This growth advantage is due to the function of the neomycin phosphotransferase gene present in the Km^r cassette introduced during the transformation to the Δ*ssrA* cells. This is demonstrated by the control Δ*crtO* strain (Fernández-González et al., 1997), which is a Km^r mutant that preserves a functional *ssrA* gene. As expected, the Δ*crtO* strain showed also a growth advantage on plates containing kanamycin, neomycin, or streptomycin (data not shown). Finally, Δ*ssrA* and wild-type cells are equally affected by other antibiotics that do not interfere with protein synthesis such as ampicillin or rifampicin (Table 1). We conclude that tmRNA is essential for growth in the presence of low concentrations of chloramphenicol, lincomycin, spiramycin, tylosin, erythromycin, and spectinomycin.

Protein synthesis is impaired in Δ*ssrA* cells pretreated with a low dose of chloramphenicol

To better define the altered resistance of Δ*ssrA* cells to the drugs mentioned in the previous section, we analyzed the wild-type and Δ*ssrA* strains for protein synthesis rate by in vivo incorporation of [^{35}S]methionine (see Materials and Methods). As shown in Figure 3A, when no antibiotic was added, the relative amount of [^{35}S]methionine incorporated into proteins was reproducibly similar in both wild-type and Δ*ssrA* strains. However, when cells were treated with 1 μg·mL^{-1} chloramphenicol for 30 min, more than a 50% reduction in [^{35}S]methionine incorporation into proteins was observed in Δ*ssrA* cells when compared to the wild-type strain (Fig. 3A). Furthermore, we could not detect any significative effect on the [^{35}S]methionine uptake (data not shown). To investigate if the translation rate of a set of proteins is more affected than other ones by chloramphenicol, we analyzed SDS/PAGE profiles of ^{35}S-labeled proteins from both wild-type and Δ*ssrA* cells treated or not with the drug. As shown in Figure 3B, no differences were found along the profiles (compare lines 1 and 3 with 2 and 4). Altogether, these results indicate that tmRNA is required for optimal protein synthesis in the presence of sublethal concentrations of chloramphenicol. This requirement is not specific for a set of proteins, therefore strongly suggesting that global impairment of protein synthesis is the primary cause of the lethality of the Δ*ssrA* strains when grown in the presence of low concentrations of protein synthesis inhibitors.

CONCLUSIONS

In this work, we have demonstrated that tmRNA is essential for survival in the presence of sublethal concentrations of several protein synthesis inhibitors. In these conditions, only a fraction of ribosomes would bind the antibiotic and becomes blocked on a mRNA. Assuming that these blocked ribosomes can recruit charged tmRNA and moreover, assuming that the first elongation cycle that incorporates the noncoded alanine is resistant to the bound antibiotic, which must be displaced, then ribosomes have the opportunity to be released. This ability is not present in Δ*ssrA* cells; therefore, blocked ribosomes accumulate, translation is impaired, and cells do not grow.

tmRNA-mediated tagging has been described in three circumstances: (1) when ribosomes reach the end of a truncated mRNA (Keiler et al., 1996), (2) when ribosomes stall at rare codons (Roche & Sauer, 1999), and (3) at natural stop codons (Roche & Sauer, 2001). In these cases, the ribosomal A-site is empty. Our results provide evidence that ribosomes stalled by some protein synthesis inhibitors can also be a substrate of tmRNA. However, in the presence of peptidyl transferase inhibitors, the ribosomal A-site can be occupied by the correct aminoacyl-tRNA or the tmRNA. In the presence of erythromycin and spectinomycin, the action of tmRNA on stalled ribosomes is more difficult to explain because peptidyl-tRNA is in the ribosomal A-site. In

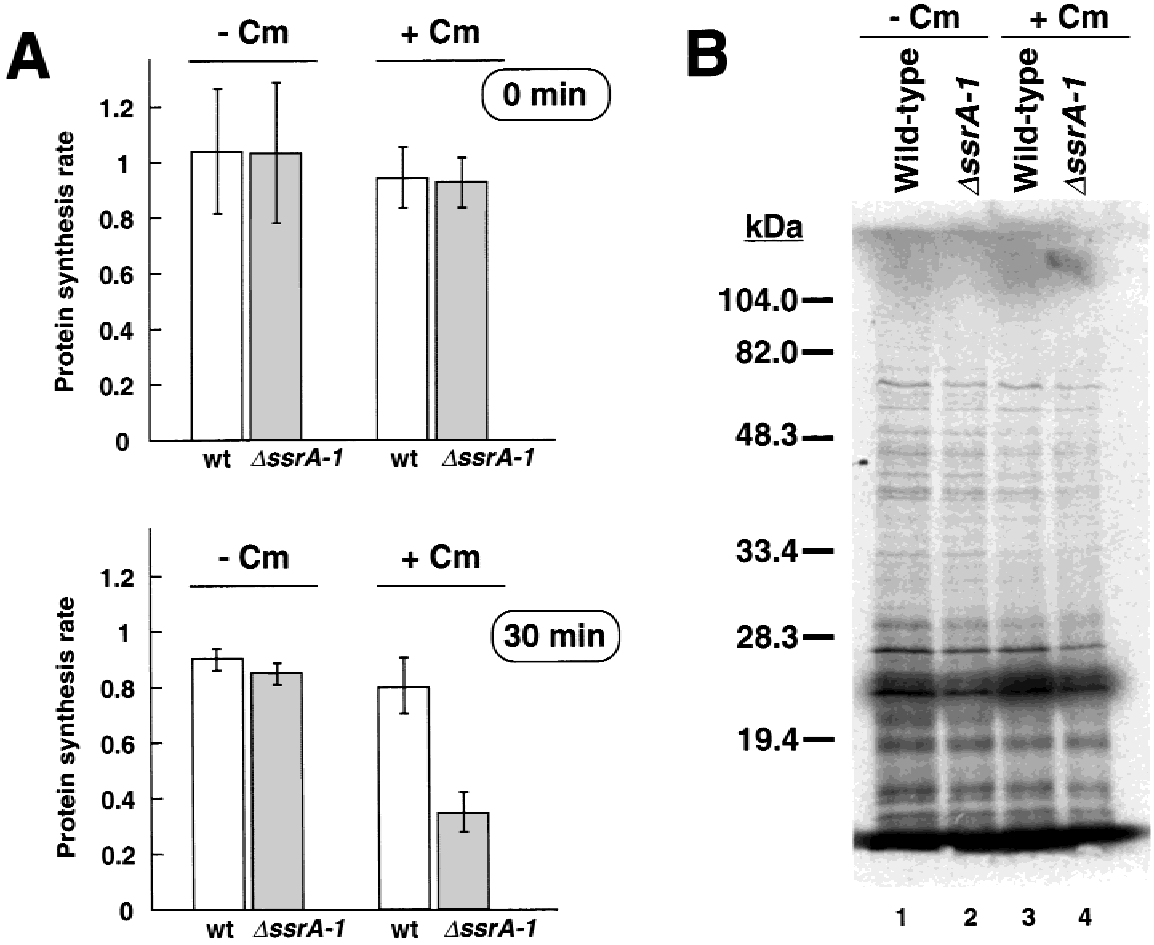

FIGURE 3. Protein synthesis is impaired in Δ*ssrA* cells. **A:** Protein synthesis rates in wild-type (wt) and Δ*ssrA* cells. Cultures (3 OD_{750} units) were incubated in the absence or presence of chloramphenicol (1 $\mu g \cdot mL^{-1}$) for 30 min. [^{35}S]methionine was added either immediately (0 min, upper panel) or 30 min after the addition of chloramphenicol (30 min, upper panel). Incubation with [^{35}S]methionine was for 15 min. The radioactivity present in the TCA-insoluble material was counted and divided by that taken up into cells. The translation rate for each strain is the average of at least three independent experiments and the standard deviations are given as bars. **B:** Detection of ^{35}S-labeled proteins in wild-type and Δ*ssrA* cells. Cultures (3 OD_{750} units) were incubated in the absence or presence of chloramphenicol (1 $\mu g \cdot mL^{-1}$) for 30 min and then with [^{35}S]methionine for 15 min. One OD_{750} unit was lysed by heating at 60 °C in Laemmli buffer. Equal amounts of radioactivity (20,000 cpm) were subjected to SDS/PAGE and ^{35}S-labeled proteins were detected by autoradiography. Size markers are indicated on the left.

any case, our data strongly suggest that tmRNA participates in a productive elongation cycle in the presence of any of the above mentioned inhibitors, most likely by displacing the antibiotic. Protein synthesis antibiotics had been and still are extremely useful tools in the dissection of the complex mechanisms of translation. Further in vivo and in vitro experiments using these antibiotics may be very useful to elucidate the mechanism of tmRNA-mediated *trans*-translation.

Finally, there is a great interest in the development of new tools against pathogenic microorganisms. Although tmRNA is not essential for growth, our results indicate that inhibition of tmRNA activity could increase the sensitivity to translation antibiotics of clinical relevance. If our results can be generalized for pathogenic bacteria, tmRNA could be an attractive target for the development of new specific drugs, as tmRNA is not present in animal cells. Thus, a combined delivery of a tmRNA inhibitor and a documented antibiotic at a lower dose would be more effective against pathogenic microorganisms.

MATERIALS AND METHODS

Strains, media, and growth conditions

Synechocystis sp. strain PCC6803 was grown in nitrate containing BG-11 medium (Rippka et al., 1979). For plates, the medium was solidified with 1% (w/v), separately autoclaved agar (Difco). Cultures were grown at 30 °C in the light (50–100 μeinsteins $\cdot m^{-2} \cdot s^{-1}$; white light fluorescent lamps), with shaking (80–100 rpm) for liquid cultures. Growth was monitored by OD_{750} or chlorophyll content that was determined as

previously described (Mackinney, 1941). Antibiotic plates were prepared by adding the drugs from stock solutions into the medium before pouring the plates. Antibiotics were used at a range of concentrations between 1 ng·mL^{-1} and the concentration at which wild-type cells die. The following antibiotics were used: ampicillin (sodium salt), chloramphenicol, erythromycin, fusidic acid (sodium salt), kanamycin sulfate, kasugamycin, lincomycin hydrochloride, neomycin, puromycin dihydrochloride, rifampicin, spectinomycin dihydrochloride, spiramycin, streptomycin sulfate, tetracycline hydrochloride, thiostrepton, and tylosin tartrate. All antibiotics except kasugamycin were of analytical grade. Kasugamycin was a generous gift from Dr. A. Jiménez. *Synechocystis* Δ*crtO* strain is a Km^r disruptant of the β-carotene ketolase gene (Fernández-González et al., 1997). *Escherichia coli* DH5α was used for recombinant DNA techniques and grown in Luria–Bertani medium with or without an appropriate antibiotic.

DNA manipulation

All recombinant DNA experiments were performed according to standard procedures (Sambrook et al., 1989). Southern blotting was done as previously described (Sambrook et al., 1989). To clone the *ssrA* gene, two primers, based on the published coordinates from the *Synechocystis* sp. PCC6803 chromosome sequence (Kaneko et al., 1996), were synthesized: 10SAF, 5′-CGCTTGTGGATCCTGTCCCAG-3′ (a *Bam*HI site is underlined) and 10SAR, 5′-TAACCGCTCGAG**TAAAGTACTGTTACTGG-**3′ (chromosome homology region is in bold type and a *Xho*I site is underlined). PCRs were performed by a standard procedure (Sambrook et al., 1989). The PCR product was cloned as a 1.5-kb *Bam*HI-*Xho*I fragment into pBluescript KS(+) (Stratagene) digested with *Bam*HI and *Xho*I to generate pBS-ssrA.

Inactivation of *ssrA* in *Synechocystis* cells

To inactivate the *ssrA* gene, pBS-ssrA was digested with *Hin*dIII and *Cla*I, then treated with Klenow DNA polymerase and dephosphorylated. The resulting blunt-ended open plasmid lacking most of the *ssrA* gene sequence was ligated to a 1.3-kb *Hin*cII fragment containing a Km^r cassette (C.K1) from Tn5 (Elhai & Wolk, 1988). Two plasmids containing the cassette in different orientation (pBS-Δ*ssrA-1* and pBS-Δ*ssrA-2*, respectively) were chosen for *Synechocystis* transformation. Cells were transformed using the method from Chauvat et al. (1986), except that cells were spread out onto nitrocellulose filters (Nucleopore REC-85, Whatman). Km^r transformants were selected on 50 μg·mL^{-1} Km-containing BG-11 plates. To facilitate segregation of the mutant chromosomes, the Km^r transformants were grown for three rounds on 100, 200, and 300 μg·mL^{-1} Km-containing BG-11 plates. Correct integration and total segregation of the mutant strains were checked by southern blot analysis. Two segregants harboring the Km^r cassette in different orientations (Δ*ssrA-1* and Δ*ssrA-2*) were chosen for further experiments.

RNA analyses

Total *Synechocystis* RNA was isolated from liquid cultures as previously described (Navarro & Florencio, 1996). Denatured RNA (5 μg) was size fractionated on 7% (w/v) polyacrylamide-8 M urea gels. RNA was transferred to and immobilized on Hybond N$^+$ nylon membranes (Amersham-Pharmacia) as previously described (Kressler et al., 1997). Probes were labeled with a DNA labeling kit (Ready to Go, Amersham-Pharmacia). Prehybridization and hybridization were done as previously described (Kressler et al., 1997).

Primer extension was done on the same RNA samples that were used for northern analysis according to García-Domínguez et al. (2000). Oligonucleotide 10SAPE (5′-CTA GGCTGCTATGGCTACC-3′) was used as a primer. To identify the positions of the primer extension stop, plasmid-borne *ssrA* was sequenced with the same primer. AMV reverse transcriptase and RNAguard were purchased from Amersham-Pharmacia.

Chloramphenicol inhibition of protein synthesis

A modified procedure from Xu et al. (2000) was used to determine protein synthesis rates; 2 μL of chloramphenicol dissolved in ethanol (1 μg·mL^{-1} final concentration) or 2 μL of ethanol were added to 1-mL cultures of *Synechocystis* wild-type and Δ*ssrA-1* strain (3 OD_{750} units) and incubated for different times under the growth conditions described above. Labeling was done using 10 μCi L-[^{35}S]methionine (1,000 Ci/mmol) for 15 min. Then, half of each sample was added to 0.5 mL ice-cold BG11 medium plus 1 mg·mL^{-1} methionine, filtered onto a Whatman GF/C paper, and washed three times with 5 mL BG11 medium; the filter was finally immersed in scintillation cocktail (= uptake sample). The remaining half of each sample was incubated with TCA and SDS [final concentrations: 10% (w/v) TCA, 1% (w/v) SDS] for 1 h at 4 °C, then filtered onto Whatman GF/C paper and washed three times with 5 mL 10% (w/v) TCA, followed by an additional wash with 5 mL acetone. The filter was finally immersed in scintillation cocktail (= incorporation sample). The radioactivity of the samples was measured by counting and quench corrected. The protein synthesis rate was expressed as the incorporation divided by the uptake to compensate for changes in label uptake among the samples.

Protein analyses

Protein concentration was determined by the Bradford method using ovoalbumin as a standard (Bradford, 1976). Protein cell extracts were prepared by adding Laemmli buffer to 3 OD_{750} units of cells and heating for 15 min at 60 °C (Ausubel et al., 1994). In vivo protein labeling experiments were performed as described above using cultures of *Synechocystis* wild-type and Δ*ssrA-1* strains. At 3 OD_{750} units, the cultures were divided in two halves; one was incubated for at least 10 min with 2 μL of chloramphenicol dissolved in ethanol (1 μg·mL^{-1} final concentration) and the other was treated with 2 μL ethanol for the same period of time. Then, 1 mL of each half was incubated with 10 μCi L-[^{35}S]methionine (1,000 Ci/mmol) for 15 min. Cells were washed twice by centrifugation with 1 mL of BG11 medium supplemented with 1 mg·mL^{-1} methionine and resuspended in 50 μL of Laemmli buffer. Labeled proteins were analyzed by SDS/PAGE (equal amounts of radioactivity was loaded in each well) followed by autoradiography.

ACKNOWLEDGMENTS

We thanks Dr. A. Jiménez (CBM, Madrid) for providing kasugamycin, L. López-Maury for help during the preparation of Figure 2, Dr. M.A. Vega-Palas for fruitful discussions, and Dr. D. Kressler (Biozentrum, Basel) for critical reading of the manuscript. J.d.l.C. acknowledges financial support from the Spanish Government (Contrato de Reincorporación, Ministerio de Educación y Cultura). This work was supported by grants from the Human Frontier Science Organization (RG291/1997), Dirección General de Enseñanza Superior (PB97-0732), and Junta de Andalucía (CVI215).

Received July 19, 2001; returned for revision August 5, 2001; revised manuscript received September 21, 2001

REFERENCES

Abo T, Inada T, Ogawa K, Aiba H. 2000. SsrA-mediated tagging and proteolysis of LacI and its role in the regulation of *lac* operon. *EMBO J 19*:3762–3769.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1994. *Analysis of proteins*, Vol. 2. New York: John Wiley & Sons, Inc.

Bilgin N, Richter AA, Ehrenberg M, Dahlberg AE, Kurland CG. 1990. Ribosomal RNA and protein mutants resistant to spectinomycin. *EMBO J 9*:735–739.

Bradford MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem 72*:248–254.

Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V. 2000. Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. *Nature 407*:340–348.

Chauvat FL, de Vries L, van der Ende A, van Arkel GA. 1986. A host-vector system for gene cloning in the cyanobacterium *Synechocystis* sp. PCC6803. *Mol Gen Genet 204*:185–191.

Elhai J, Wolk CP. 1988. A versatile class of positive-selection vectors based on the nonviability of palindrome-containing plasmids that allows cloning into long polylinkers. *Gene 68*:119–138.

Fernández-González B, Sandmann G, Vioque A. 1997. A new type of asymmetrically acting β-carotene ketolase is required for the synthesis of echinenone in the cyanobacterium *Synechocystis* sp. PCC6803. *J Biol Chem 272*:9728–9733.

Gabashvili IS, Gregory ST, Valle M, Grassucci R, Worbs M, Wahl MC, Dahlberg AE, Frank J. 2001. The polypeptide tunnel system in the ribosome and its gating in erythromycin resistance mutants L4 and L22. *Mol Cell 8*:181–188.

Gale EF, Cundliffe E, Reynolds PE, Richmond MH, Waring MJ. 1981. Antibiotic inhibitors of ribosome function. In: *The molecular basis of antibiotic action*. London: John Wiley and Sons. pp 402–547.

García-Domínguez M, Muro-Pastor MI, Reyes JC, Florencio FJ. 2000. Light-dependent regulation of cyanobacterial phytochrome expression. *J Bacteriol 182*:38–44.

Gottesman S, Roche E, Zhou Y, Sauer RT. 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes & Dev 12*:1338–1347.

Huang C, Wolfgang MC, Withey J, Koomey M, Friedman DI. 2000. Charged tmRNA but not tmRNA-mediated proteolysis is essential for *Neisseria gonorrhoeae*. *EMBO J 19*:1098–1107.

Hutchison CA, Peterson SN, Gill SR, Cline RT, White O, Fraser CM, Smith HO, Venter JC. 1999. Global transposon mutagenesis and a minimal *Mycoplasma* genome. *Science 286*:2165–2169.

Jain SK, Gurevitz M, Apirion D. 1982. A small RNA that complements mutants in the RNA processing enzyme ribonuclease P. *J Mol Biol 162*:515–533.

Kaneko T, Sato S, Kotani H, Tanaka A, Asamizu E, Nakamura Y, Miyajima N, Hirosawa M, Sugiura M, Sasamoto S, Kimura T, Hosouchi T, Matsuno A, Muraki A, Nakazaki N, Naruo K, Okumura S, Shimpo S, Takeuchi C, Wada T, Watanabe A, Yamada M, Yasuda M, Tabata S. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res 3*:109–136.

Karzai AW, Roche ED, Sauer RT. 2000. The SsrA-SmpB system for protein tagging, directed degradation and ribosome rescue. *Nat Struct Biol 7*:449–455.

Karzai AW, Sauer RT. 2001. Protein factors associated with the SsrA-SmpB tagging and ribosome rescue complex. *Proc Natl Acad Sci USA 98*:3040–3044.

Karzai AW, Susskind MM, Sauer RT. 1999. SmpB, a unique RNA-binding protein essential for the peptide-tagging activity of SsrA (tmRNA). *EMBO J 18*:3793–3799.

Keiler KC, Waller PRH, Sauer RT. 1996. Role of peptide tagging system in degradation of protein synthesized from damaged messenger RNA. *Science 271*:2068–2081.

Knudsen B, Wover J, Zwieb C, Gorodkin J. 2001. tmRDB (tmRNA database). *Nucleic Acids Res 29*:171–172.

Komine Y, Kitabatake M, Yokogawa T, Nishikawa K. 1994. A tRNA-like structure is present in 10Sa RNA, a small stable RNA from *Escherichia coli*. *Proc Natl Acad Sci USA 91*:9223–9227.

Kressler D, de la Cruz J, Rojo M, Linder P. 1997. Fal1p is an essential DEAD-box protein involved in 40S-ribosomal-subunit biogenesis in *Saccharomyces cerevisiae*. *Mol Cell Biol 17*:7283–7294.

Levchenko I, Seidel M, Sauer RT, Baker TA. 2000. A specificity-enhancing factor for the ClpXP degradation machine. *Science 289*:2354–2356.

Mackinney G. 1941. Absorption of light by chlorophyll solutions. *J Biol Chem 140*:315–322.

Muto A, Fujihara A, Ito K-I, Matsuno J, Ushida C, Himeno H. 2000. Requirement of transfer-messenger RNA for the growth of *Bacillus subtilis* under stresses. *Genes Cells 5*:627–635.

Muto A, Ushida C, Himeno H. 1998. A bacterial RNA that functions as both a tRNA an mRNA. *Trends Biochem Sci 23*:25–29.

Navarro F, Florencio FJ. 1996. The cyanobacterial thioredoxin gene is required for both photoautotrophic and heterotrophic growth. *Plant Physiol 111*:1067–1075.

Nissen P, Hansen J, Ban N, Moore PB, Steitz TA. 2000. The structural basis of ribosome activity in peptide bond formation. *Science 289*:920–929.

Poulsen SM, Kofoed C, Vester B. 2000. Inhibition of the ribosomal peptidyl transferase reaction by the mycarose moiety of the antibiotics carbomycin, spiramycin and tylosin. *J Mol Biol 304*:471–481.

Rippka R, Deruelles J, Waterbury JB, Herdman M, Stanier RY. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. *J Gen Microbiol 111*:1–61.

Roche ED, Sauer RT. 1999. SsrA-mediated peptide tagging caused by rare codons and tRNA scarcity. *EMBO J 18*:4579–4589.

Roche ED, Sauer RT. 2001. Identification of endogenous SsrA-tagged proteins reveals tagging at positions corresponding to stop codons. *J Biol Chem 276*:28509–28515.

Rodríguez-Fonseca C, Amils R, Garret RA. 1995. Fine structure of the peptidyl transferase center on 23S-like rRNAs deduced from chemical probing of antibiotic-ribosome complexes. *J Mol Biol 247*:224–235.

Rudinger-Thirion J, Giegé R, Felden B. 1999. Aminoacylated tmRNA from *Escherichia coli* interacts with prokaryotic elongation factor Tu. *RNA 5*:989–992.

Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: A laboratory manual, 2nd ed., Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Tadaki T, Fukushima M, Ushida C, Himeno H, Muto A. 1996. Interaction of 10Sa RNA with ribosomes in *Escherichia coli*. *FEBS Lett 399*:223–226.

Tous C, Vega-Palas MA, Vioque A. 2001. Conditional expression of the RNase P from the cyanobacterium *Synechocystis* 6803 allows detection of precursor RNAs. Insights in the in vivo maturation pathway of transfer and other stable RNAs. *J Biol Chem 276*:29059–29066.

Vioque A. 1992. Analysis of the gene encoding the RNA subunit of ribonuclease P from cyanobacteria. *Nucleic Acids Res 20*:6331–6337.

Wassarman KM, Zhang A, Storz G. 1999. Small RNAs in *Escherichia coli*. *Trends Microbiol 37*:37–45.

Watanabe T, Sugita M, Sugiura M. 1998. Identification of 10Sa RNA (tmRNA) homologues from the cyanobacterium *Synechococcus* sp. strain PCC6301 and related organisms. *Biochim Byophys Acta 1396*:97–104.

Withey J, Friedman D. 1999. Analysis of the role of *trans*-translation in the requirement of tmRNA for λ*imm*P22 growth in *Escherichia coli*. *J Bacteriol 181*:2148–2157.

Xu Y, Mori T, Johnson CH. 2000. Circadian clock-protein expression in cyanobacteria: Rhythms and phase setting. *EMBO J 19*:3349–3357.

Increased sensitivity to protein synthesis inhibitors in cells lacking tmRNA.

J de la Cruz and A Vioque

RNA 2001 7: 1708-1716