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Activity of Translation System and Abundance of tmRNA during Development of *Streptomyces aureofaciens* Producing Tetracycline

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ABSTRACT. Transition from exponential phase of growth to stationary phase in *Streptomyces aureofaciens* is characterized by a decrease in the rate of translation and induction of tetracycline (Ttc) biosynthesis. In exponential phase, no significant changes were found in the activity of ribosomes at binding of ternary complex Phe-tRNA.EF-Tu.GTP to the A-site on ribosomes. Overexpression of Ttc in stationary phase is accompanied by a decrease in the binding of the ternary complex Phe-tRNA.EF-Tu.GTP to the A-site of ribosome and a formation of an aggregate with Ttc by part of the ribosomes. Antibiotics that cause ribosome to stall or pause could increase the requirement for tmRNA in the process called *trans*-translation. We found differences in the level of tmRNA during the development of *S. aureofaciens.* Subinhibitory concentrations of Ttc, streptomycin and chloramphenicol induced an increase in the tmRNA level in cells from the exponential phase of growth. *In vitro trans*-translation system of *S. aureofaciens* was sensitive to Ttc at a concentration of >15 μmol/L; the *trans*-translation system can thus be considered to contribute to resistance against Ttc produced only at sublethal concentrations. These experiments suggest that the main role of the rising tmRNA level at the beginning of the Ttc production is connected with ribosome rescue.

Abbreviations

Clm	chloramphenicol	Stm	streptomycin	DTT	1,4-dithiothreitol
Kir	kirromycin	Ttc	tetracycline	tmRNA	transfer-messenger RNA

Streptomycetes are soil microorganisms, exposed to various physical and chemical conditions including stresses that activate specialized responses such as synthesis of antibiotics and morphological differentiation from vegetative cells to aerial mycelium and spores. Many antibiotics produced by streptomycetes are known inhibitors of protein synthesis. The ribosome plays a central role in sensing and adaptation to environmental stress induced by the presence of inhibitors of ribosomal functions (van Bogelen and Neidhardt 1999). Ttc inhibits protein synthesis by interfering with the binding of aminoacyl-tRNA to the A-site of ribosome (Suarez and Nathans 1965). Crystallographic studies of Ttc–ribosome complexes (Brodersen *et al.* 2000; Pioletti *et al.* 2001) revealed six Ttc-binding sites on 30S subunits. The binding site localized between the distorted minor groove of H34 and the stem-loop of H31 is responsible for the blockage of the A-site. There are two mechanisms of ribosomal resistance to Ttc. One is mediated by ribosomal protection proteins (Roberts 1996), the other by the mutation 1058G→C on 16S RNA (Ross *et al.* 1998). Resistance mediated by ribosome protection proteins (Tet-family) confers resistance only at a low concentration of Ttc (Dantley *et al.* 1998).

Experiments with *Synechocystis* showed that in the presence of subinhibitory concentrations of protein synthesis inhibitors this cyanobacterium is dependent on the presence of tmRNA (de la Cruz and Vioque 2001). Preincubation of *ssrA* mutant *Synechocystis* with Clm led to a 50 % reduction in protein synthesis relative to the level of translation observed in wild-type *Synechocystis* with Clm. These results suggest that tmRNA is required for efficient translation in the presence of protein synthesis inhibitors (Vioque and de la Cruz 2003). When a ribosome translates to the 3′ end of an incomplete mRNA or the mRNA lacking a stop codon, tmRNA is charged with alanine by alanyl-tRNA synthetases and alanine enters the A-site of the ribosome, which is then transferred to the stalled polypeptide chain (Keiler *et al.* 2000). tmRNA is a hybrid of a tRNA-like domain and an mRNA-like domain connected by pseudoknots. Translation resumes at an inter-

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nal position in tmRNA but not on the mRNA on which the ribosomes were stalled. This co-translation reaction terminates at the stop codon. The final translation product is a chimeric polypeptide in which the specific tagged polypeptide is recognized and degraded by ATP-dependent proteinases (Herman 1998). The read-through of stop codons by ribosomes can lead to expression of potentially toxic extended proteins. Production of tmRNA tagged proteins in *E. coli* has been observed to increase in the presence of both suppressor tRNA and the miscoding drugs kanamycin and Stm (Abo *et al.* 2002) which cause translational read-through of stop codons. tmRNA has been shown to be essential for growth in *Neisseria gonorrhoeae* (Huang *et al.* 2002), *Mycoplasma* species (Hutchison *et al.* 1999) and for invasion of macrophages by *Salmonella enterica* (Julio *et al.* 2000). In *Caulobacter crescentus* it is required for normal timing of the G_1-to-S transition. A deletion of the *ssrA* gene or the gene encoding small basic protein SmpB, which is required for association of tmRNA with ribosome (Karzai *et al*. 1999), results in a specific delay in the cell cycle (Keiler and Shapiro 2003). In *Bacillus subtilis*, tmRNA is thermoinducible, and its ability to recycle stalled ribosomes *via trans*-translation is involved in the stress tolerance of the cell (Muto *et al.* 2000). Furthermore, cells lacking a functional tmRNA exhibit a variety of subtle phenotypes such as slow growth at a high temperature, reduced motility and inhibition of phage growth and enhanced activity of several repressors (Slavcev and Hayes 2004; Munavar *et al.* 2005).

The presence of a functional *trans*-translational system is important to allow the survival of several species of bacteria under unfavorable conditions.

Ribosomes stalled at the truncated mRNA lacking a termination codon are released and the tmRNA system facilitates the degradation of the truncated mRNAs and allows 3′ to 5′ exonucleases to access the free mRNA 3′ end (Ueda *et al*. 2002). Thus, the tmRNA quality control mechanism not only degrades aberrant polypeptides once produced, but also prevents the production of aberrant polypeptides through a rapid elimination of damaged mRNAs. In addition, *trans*-translation system is activating during the translation of full-length mRNA when ribosomes pause at rare codons or stop codons because the cognate tRNA or release factor is restricted (Roche and Sauer 1999; Collier *et al.* 2002; Hayes *et al.* 2002; Li *et al*. 2005). Proteomic studies have shown that many proteins including transcription factors are tagged by tmRNA. The regulatory role of tmRNA was recognized in many studies with mutants of *ssrA* genes.

Despite the important biological function of tmRNA, its physiological role in antibiotic-producing streptomycetes remains unresolved. Here we investigated a possible role of the tmRNA during development of streptomycetes producing Ttc. We addressed three basic questions: (*1*) Is the level of tmRNA regulated during the growth and development in *Streptomycetes aureofaciens*? (*2*) How does the presence of subinhibitory concentrations of antibiotic influence the abundance of the tmRNA? (*3*) Is *trans*-translation system involved in the mechanism of resistance to the produced drug? Our data show differences in abundance of tmRNA due to the presence of various antibiotics interfering with protein synthesis.

MATERIALS AND METHODS

Streptomyces aureofaciens strain MBU 84/25 was obtained from the collection of microorganisms of the *Institute of Microbiology* (*Acad. Sci. Czech Rep.*). Spores were prepared according to Kieser *et al*. (2000) and used for inoculation of MJ complex medium (in g/L): casamino acids 10, glycerol 10, yeast extract 2, $(NH_4)_2SO_4$ 2, K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.2 and soya extract 10 mL (pH 7.2). Cells from different stages of development were harvested by centrifugation and washed with the standard buffer (in mmol/L): Tris-HCl 20 (pH 7.6), NH_4Cl 40, $MgCl_2$ 10, DTT 1, phenylmethanesulfonyl fluoride (PMSF) 1. Cultures were grown on reciprocal shaker (2.3 Hz) at 28 °C.

Isolation of RNA from spores and vegetative cells. Spores or vegetative cells (0.6 g wet material) were mixed with RNA Blue containing guanidium thiocyanate, sodium citrate, 2-mercaptoethanol, Na_3VO_4 (*Top-Bio*) and disrupted with glass beads in FastPrep homogenizer (*Qbiogene Inc*.). Homogenate (1 mL) was mixed with 50 μL of 10 % sarkosyl and incubated for 5 min at room temperature. The sample was shaken with 0.2 mL of chloroform, and centrifuged (14 500 ***g***, 10 min, 4 °C). The water phase containing RNA was taken off and precipitated by the mixture (the volume corresponded to the above water phase) of sodium acetate (pH 5.5)–2-propanol (0.1 : 1, *V*/*V*) at 20 °C. RNA was sedimented at 14 500 ***g*** (10 min, 4 °C) and washed with 1 mL 75 % ethanol. The final sediment was solubilized in a sterile 10 mmol/L Tris-HCl (pH 8). RNA was analyzed in 7 % polyacrylamide with 6 mol/L urea sequencing gels and visualized with ethidium bromide.

Analysis of tmRNA by Northern hybridization. Aliquots of the total RNA (50 μg) were separated by electrophoresis in 7 % polyacrylamide gels with 6 mol/L urea. A nylon membrane was blotted with separated RNA using a vacuum blotter Hybaid. ^{32}P-Labeled *ssrA* probe was added for hybridization for 16 h at

48 °C. The blot was washed with 0.5× SSC and visualized by PhosphorImager. The relative amounts of tmRNA were quantified using an Aida Image analyzer.

Cloning of the ssrA *gene of* S. aureofaciens. The gene encoding tmRNA from *S. aureofaciens* was isolated from the total RNA of spores or vegetative cells by reverse transcription and PCR amplification of DNA using an upstream primer (5′-GGG GAT GAT CGG TTT CGA CAG-3′) and the downstream primer (5′-TGG TGG AGA TGG CGG GAA TC-3′). Plasmid with the *ssrA* gene under the control of the T7 promoter was constructed by PCR amplification of *S. aureofaciens* genomic DNA with upstream primer (5′-CGA ATT CTA ATA CGA CTC ACT ATA GGG GGA TGA TCG GTT TCG ACA G-3′) containing an *Eco*RI restriction site and T7 promoter sequences. The amplified DNA was digested with *Eco*RI and cloned into pGEM-T Easy vector to generate pGEM-SaT7.

Sequencing analysis. For sequencing, the amplified PCR fragments were extracted from agarose gels using the MinElute gel extraction kit (*Qiagen*, Germany). Purified PCR fragments were sequenced with the ABI Prism BigDye terminator v3.1 cycle sequencing kit (*Applied Biosystems*, UK). The chain termination reaction (Sanger *et al.* 1977) was performed by cycle-sequencing technique (Murray 1989) according to the manufacturer's protocol. The sequence was determined with an ABI Prism 3100 DNA sequencer (*Applied Biosystems*), an ally edited and analyzed with the Lasergene® sequence analysis software (*DNAStar*, USA) (the *GenBank* accession number for the *ssrA* of *S. aureofaciens* is AY 616521).

Preparation of S30 fraction and sedimentation analysis. Cells were disrupted with glass beads in FastPrep homogenizer and extracted with standard buffer (mmol/L): Tris-HCl 10 (pH 7.6), NH_4Cl 60, magnesium diacetate 8, DTT 1 and 1 μg/mL DNAase RQ1 (RNAase-free). Extract was centrifuged (15 000 ***g***, 30 min, 4 °C); the supernatant was again centrifuged (30 000 ***g***, 30 min) to obtain the S30 fraction. Samples containing 2 A_{260} units of the S30 fractions were analyzed by centrifugation (rotor SW 50.1; 834 Hz, 100 min, 4 °C) in 5 mL linear (5–22 %) sucrose gradients.

Binding of ternary complex ^{14}*C-L-Phe-tRNA.EF-Tu.GTP to ribosomes.* The complex EF-Tu.GTP was prepared in the reaction mixture (100 μL) containing (mmol/L) Tris-HCl 50 (pH 7.5), magnesium diacetate 10, NH_4Cl 60, GTP 5, EF-Ts 120, phosphoenolpyruvate 40; 10 μg pyruvate kinase nd 600 pmol EF-Tu from *S. aureofaciens* (Mikulík *et al.* 1983); the incubation was done for 10 min at 30 °C. Aliquots (100 μL) containing 50 mmol/L Tris-HCl (pH 7.6), 8 mmol/L magnesium diacetate, 80 μg poly(U), 25 pmol ribosomes were preincubated with 50 pmol of deacylated Phe-tRNA for 30 min at 30 °C. Then 120 pmol of EF-Tu.GTP and 200 pmol ^{14}C-L-Phe-tRNA (1500 pmol per A_{260} unit) were added and incubation continued for another 10 min at 30 °C. Reactions were terminated by addition of 3 mL of cold buffer (mmol/L: Tris-HCl 5, pH 7.6; NH_4Cl 100, magnesium diacetate 8). Radioactivity of Phe-tRNA bound to the A-site of ribosomes was determined by membrane filter assay.

In vitro trans-*translation using the stalled complex of ribosomes.* The composition of the reaction mixture was similar as described by Himeno *et al.* (1997), Konno *et al.* (2004) and Asano *et al.* (2005). Polyphenylalanine was synthesized in 100 μL reaction mixtures containing (mmol/L): Tris-HCl 50 (pH 7.6), magnesium diacetate 10, NH_4Cl 80, DTT 1, phosphoenol pyruvate 5, ATP 1, GTP 0.5, and 15 pmol L-phenylalanine, 2 A_{260} units of S30 fraction and 100 μg poly(U). The time dependent incorporation of ^{3}H-L-phenylalanine (15 pmol) into polyphenylalanine was examined in a parallel experiment. After a 15-min incubation at 32 °C, phenylalanine incorporation was saturated. To the stalled complex of ribosomes was added the mixture of (μmol/L): each of *U*-^{14}C-L-alanine 20, *U*-^{14}C-L-arginine 20, *U*-^{14}C-L-leucine 20, *U*-^{14}C-L-lysine 20, and 50 μmol/L each of remaining unlabeled amino acids. After a 15-min incubation at 32 °C, the reaction was terminated by adding trichloroacetic acid to a final concentration of 5 %. Material insoluble in hot trichloroacetic acid was filtered over *Whatman* GF/C filters and assayed for radioactivity.

RESULTS

Protein synthesis, biosynthesis of tetracycline and tmRNA abundance. To obtain convincing evidence about the presence of tmRNA in aerial spores and vegetative cells total RNA of *S. aureofaciens* was isolated and separated; then Northern blot was probed with PCR-amplified DNA of *S. aureofaciens* with primers derived from conservative 3′ and 5′ ends of *S. coelicolor ssrA*. Two spots ≈380 and 460 bp were detected by hybridization. Spot 380 bp (Fig. 1) from spore RNA was eluted and used for reverse transcription and cDNA was subjected to sequence analysis. The primers were also employed for the preparation of *ssrA* by PCR amplification of the DNA from vegetative cells. The results of sequence analysis showed that *ssrA* from aerial spores and vegetative cells have identical structure. The gene consists of 382 bp, the characteristic tag-reading frame located at the positions 98–140. The complete nucleotide sequence is deposited in *GenBank* AY616521. The data are the first report on the presence of tmRNA in the aerial spores.

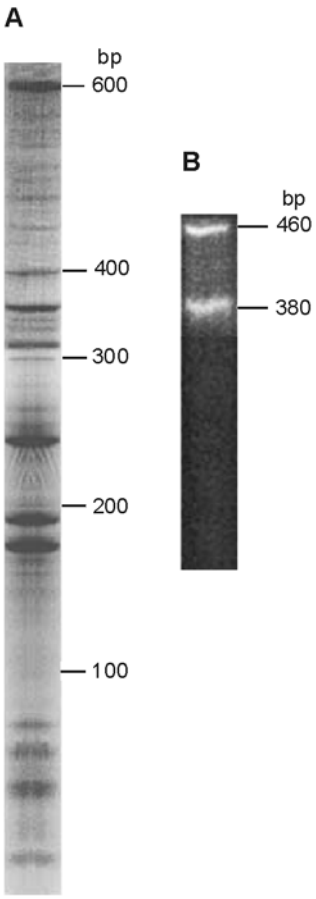

Fig. 1. Analysis of total RNA from spores of *S. aureofaciens* producing tetracycline. Aerial spores were disintegrated with glass beads in the presence of RNA-Blue and RNA was analyzed in 7 % polyacrylamide–6 mol/L urea gel. **A**: part of the gel containing RNAs <600 bp was visualized with ethidium bromide, and Northern blot (**B**) was probed with ^{32}P-labeled PCR amplified DNA using oligonucleotides derived from conservative sequences of *S. coelicolor ssrA*; spot of 380 bp was identified as tmRNA and the second hybridizable spot of 460 bp was considered to be a precursor tmRNA.

The information on the rate of protein synthesis, activity of ribosomes and production of Ttc was considered to be essential for studying the potential role of tmRNA in the development of *S. aureofaciens*. The rate of protein synthesis was measured (at time intervals the cells were pulse-labeled with *U*-^{14}C-L-leucine); growth of the culture in the complex medium (Fig. 2A) was characterized by a rapid increase in ^{14}C-leucine incorporation up to 24 h, thereafter the rate decreased. After a 96-h cultivation, the decline in the rate of protein synthesis was ≈75 %. The synthesis of Ttc started from 16 h and, after 120 h of cultivation, the Ttc concentration reached about 2 mg/mL. To test whether the decline in the rate of protein synthesis during transition from exponential phase of growth (up to 24 h) to antibiotic-producing phase (stationary phase) is connected with the activity of ribosomes, binding of the ternary complex ^{14}C-Phe-tRNA.EF-Tu.GTP to the A-site on ribosomes was examined. As shown in Fig. 3, subinhibitory concentration of Ttc (20–100 μg/mL) had no effect on the activity of ribosomes isolated after 14 and 24 h of cultivation. When Ttc concentration in the cultivation medium reached >400 μg/mL, ribosomes isolated from 32-, 48- and 72-h cultures were less active in the binding of the ternary complex to the A-site. We also determined the distribution of ribosomes and ribosomal subunits in S30 fractions during development (Fig. 4; these experiments were performed with cells washed several times to remove contamination with Ttc). The major part of ribosomal population isolated from 24-h cultures contained 70S ribosomes. The amount of ribosomal subunits in S30 fractions increased after 32 h of cultivation and, after 72 h, part of ribosomes formed aggregates (that sediment at the bottom of centrifugation tube) with Ttc. The aggregates from several tubes were collected and extracted with chloroform. Spectrometric analysis (measuring maximum absorbance at 220, 268 and 355 nm) confirmed the presence of Ttc in the aggregates. These data indicate that accumulation of Ttc in the cultivation medium causes the inactivation of ribosomal function and leads to changes in the distribution of ribosomes.

Translation pausing can lead to cleavage of the A-site codon and facilitate conscription of the tmRNA-quality-control system to suffering ribosomes. To follow the abundance of the tmRNA during development and differentiation, cells from different stages of growth and differentiation were used to isolate total RNA in the presence of the RNAase inhibitor; after analyzing and probing the ^{32}P-labeled *ssrA*, the radioactive spot corresponding to 380 bp (tmRNA) was quantified. The level of tmRNA increased up to 48 h of cultivation and then, during the late stationary phase, decreased (Fig. 2B); in controls, the level of 5S RNA was monitored to eliminate general loss of RNA. The results showed no substantial change in 5S RNA level during the experiment. The decrease in tmRNA during the late exponential phase in *S. aureofaciens* suggests that the tmRNA was degraded. To support these observations the half-life of tmRNA from the exponentially growing cells of *S. aureofaciens* (14-h culture) and from the stationary-phase cells (68-h culture) was determined by inhibition of transcription with rifamycin and the decay of tmRNA was measured (Fig. 5). tmRNA from exponentially growing cells was stable for >50 min; in cells from late stationary phase ≈50 % was degraded during 1 h.

Involvement of tmRNA in resistance to tetracycline. We determined whether subinhibitory concentrations of Clm, Kir, Stm and Ttc affect the level of tmRNA. The outcome of this experiment is that induction of tmRNA expression under conditions where Ttc has been added that is naturally synthesized is instantaneous and remains constant (Table I). The most pronounced effect on the increase of the tmRNA was observed with Stm, which caused progressive enhancement of the tmRNA levels (>3×). Clm also exhibited a significant effect on the rise in tmRNA concentration. In contrast, Kir had only a low influence on the tmRNA abundance.

To examine the sensitivity of the *trans*-translation system to Ttc, stalled complexes of ribosomes with poly(U) were prepared from the 24-h-culture S30 fraction (*see Materials and Methods*). The polyphenyl-

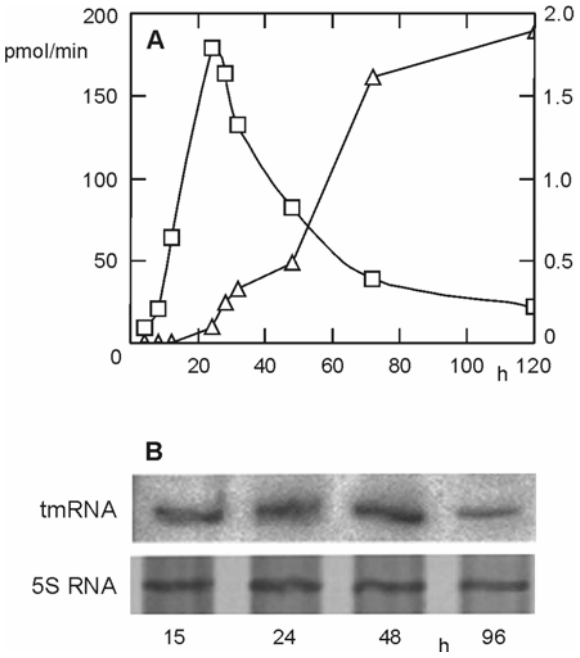

Fig. 2. Protein synthesis and production of tetracycline in *Streptomyces aureofaciens* (**A**) and analysis of tmRNA during development of *S. aureofaciens* (**B**). **A**: The rate of protein synthesis was examined using pulse-label experiments: at time intervals, samples (5 mL) were pulse-labeled for 5 min with *U*-^{14}C-L-leucine (583 TBq/mol); two 1-mL samples were taken, precipitated with 1 mL 10 % trichloroacetic acid and incubated for 20 min at 95 °C; precipitates were collected on glass micro fiber filters, washed with 5 % trichloroacetic acid, dried and measured for radioactivity; *squares* – rate of protein synthesis in complex medium, *triangles* – synthesis of tetracycline. Data are average values from 3 parallel experiments; the error limits were within ±5 %. **B**: Aliquot samples of cultures were taken after 15-, 24-, 48- and 96-h cultivation; total RNA was isolated and 50 μg RNA samples were separated by gel (7 % polyacrylamide–6 mol/L urea) electrophoresis; tmRNA was detected by Northern hybridization with ^{32}P-labeled probe. The amount of tmRNA was quantified by Aida Image analyzer; the blots were re-probed for 5S RNA.

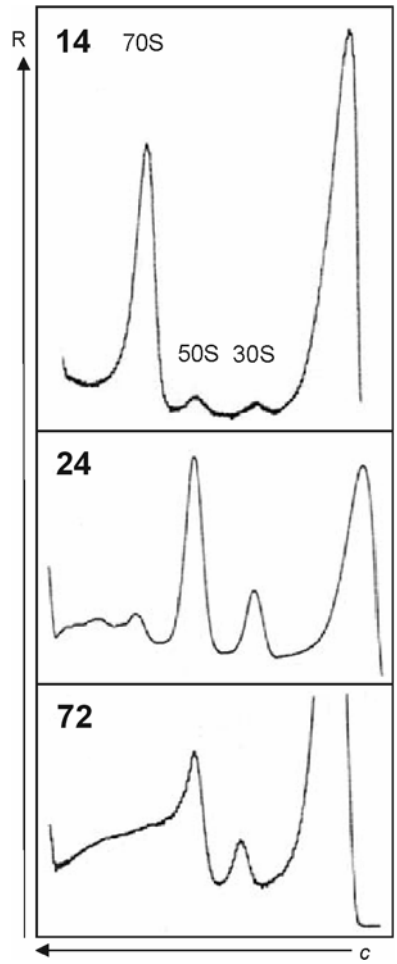

Fig. 4. Sedimentation analysis of ribosomes from *S. aureofaciens*. S30 fractions from **14**-, **24**- and **72**-h cells growing on complex medium and producing tetracycline; sedimentation is from *right* to *left*; R – detector response, *c* – sucrose concentration.

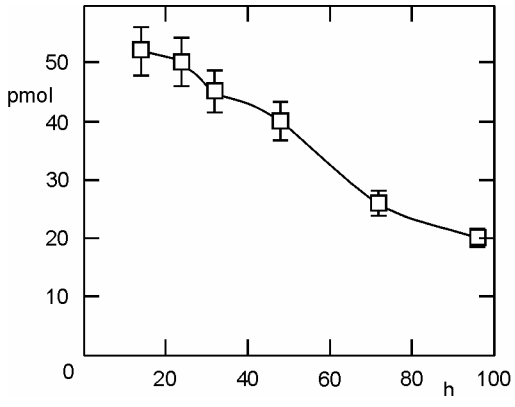

Fig. 3. Binding of ternary complex ^{14}C-L-Phe-tRNA.EF-Tu.GTP to ribosomes isolated after 15-, 24-, 48- and 72-h tetracycline-producing cultures was performed at 30 °C, close to optimum temperature for growth of *S. aureofaciens*; the results correspond to the average of 3 experiments; the error limits were within ±4 %.

alanine incorporation was saturated after a 15-min incubation; the stalled complex of ribosomes (mixed with ^{14}C-labeled amino acids, which are constituents of the tag-peptide of *S. aureofaciens* tmRNA) was resistant to the presence of ≤15 μmol/L Ttc; its higher concentration inhibited the incorporation of ^{14}C-amino acids to tag-peptides (Fig. 6). These data indicate that *S. aureofaciens* responds to the presence of subinhibitory concentrations of Ttc by elevating the tmRNA level. The increased concentration of Ttc causing inhibition of *trans*-translation thus indicates that the *trans*-translation system may contribute to the resistance against the drug produced only at sublethal concentrations.

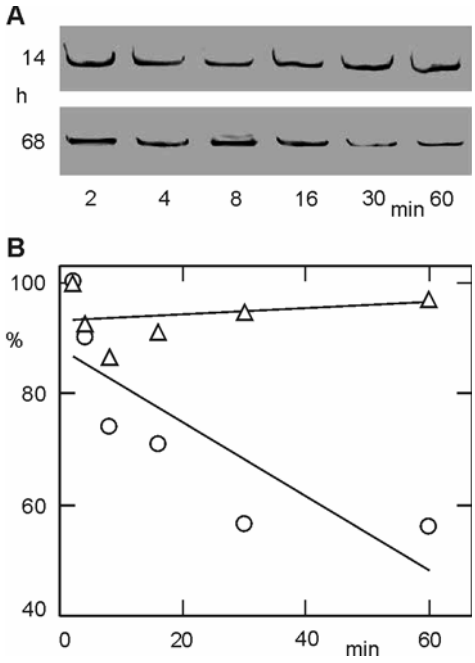

Fig. 5. Northern-blot analysis of stability of the tmRNA from 14- and 68-h culture of *S. aureofaciens*. Representative blot analysis of the tmRNA expression; transcription was inhibited by addition of rifamycin (300 μg/mL) and aliquots were taken at the indicated time for RNA extraction and Northern analysis (for quantification of tmRNA *see* Fig. 2B). The value obtained at time zero was used as 100 %; the amount of tmRNA remaining after addition of rifamycin was plotted as a function of time; the half-life was determined by linear regression.

Table I. The effect of antibiotics (10-min, 1- and 3-h treatment)[a] on the level of tmRNA (%)[b] in *S. aureofaciens*

Antibiotic	10 min	1 h	3 h
Clm	105 ± 9.4	113 ± 15.0	150 ± 11.8
Kir	102 ± 12.1	104 ± 11.4	111 ± 14.9
Stm	120 ± 9.4	174 ± 17.2	312 ± 26.9
Ttc	132 ± 15.0	138 ± 17.2	130 ± 17.1

[a]Clm (8 μg/mL), Kir (5), Stm (5), or Ttc (200) were added to exponentially growing cultures; at time intervals, total RNA was isolated; aliquot parts were analyzed by electrophoresis in polyacrylamide–urea gels; Northern blots were hybridized with ^{32}P-labeled probe.

[b]Control (100 %) – the level of tmRNA before the addition of antibiotics; values are averages from three experiments.

DISCUSSION

The rate of protein synthesis is regulated, thereby integrating the translation process with other metabolic pathways of the cell. Altered translation rates occur under various conditions including stress, during the transition from active growth to antibiotic producing phase and cell differentiation in streptomycetes. At present, conclusive evidence is not available about how streptomycetes control the transition from primary metabolism and activate secondary metabolism. We examined the rate of protein synthesis during the development of Ttc-producing strain of *S. aureofaciens*, activity of ribosomes, abundance of tmRNA and sensitivity of *trans*-translation to Ttc. We showed that the translational system is sensitive to the accumulation of Ttc; it changes the pattern of ribosomes and the activity of ribosomes at the binding of ternary complex Phe-tRNA.EF-Tu.GTP to the A-site on ribosomes. Antibiotics that cause ribosome to stall or pause could increase the cleavage of mRNA within or in adjacent to the A-site codon. The A-site mRNA-cleavage pathway accompanied with pausing ribosomes and induction of the tmRNA system can reduce translational errors and production of aberrant polypeptides (Hayes and Sauer 2003). The binding of Ttc blocks the binding of the ternary complex to the A-site by sterically interfering with accommodation of the aminoacyl-tRNA (Suarez and Nathans 1965). Over-expression of Ttc at the late-stationary phase of growth leads to the reduction of translation rate and to the decrease in the tmRNA level. The *trans*-translation system may then contribute to the survival of streptomycetes only in the presence of subinhibitory concentration of Ttc.

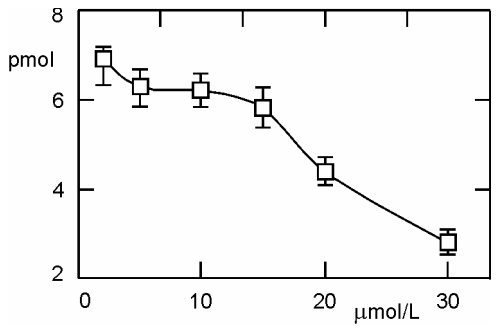

Fig. 6. Effect of tetracycline on *in vitro* incorporation of amino acids by stalled complex of ribosomes. Preincubated S30 fractions ($2\,A_{260}$ units) were used for translation of poly(U) in reaction mixtures (100 μL) containing (mmol/L) Tris-HCl 50 (pH 7.6), NH_4Cl 80, $MgCl_2$ 10, phenylalanine 15, phosphoenolpyruvate 5, DTT 1, ATP 1, GTP 0.5, and 100 μg poly(U). After a 15-min incubation, phenylalanine incorporation was saturated. To the stalled complex of ribosomes was added 20 μmol/L each of *U*-^{14}C-L-alanine, *U*-^{14}C-L-arginine, *U*-^{14}C-L-leucine, *U*-^{14}C-L-lysine, and 50 μmol/L each of remaining unlabeled amino acids and tetracycline (for further details *see* also *Materials and Methods*); the values are the averages from four independent experiments.

Our data suggest that the main role of the rising level of tmRNA at the beginning of Ttc production is connected with ribosome rescue. Ribosome release mediated by *trans*-translation becomes more critical for organisms under stress conditions. This suggestion is supported by experiments when the level of tmRNA increased in the presence of Ttc and other antibiotics interfering with protein synthesis. A report on *ssrA* in *Streptomyces lividans* (Braud *et al.* 2005) showed that disruption of *ssrA* locus has no effect on the morphology of mutant colonies. The *ssrA* gene was constitutively expressed during cultivation in liquid medium. On the other hand, we found differences in the abundance of tmRNA during the development of *S. aureofaciens* – our data show differences in the tmRNA stability during development (*cf.* Fig. 6). As tmRNA is stable during the exponential phase (half-life >50 min) while in the stationary phase about ½ tmRNA is degraded, it remains to be established how the stability of tmRNA is regulated. Hong *et al*. (2005) showed that SmpB protein (small protein B) protects tmRNA from degradation. Accommodation of tmRNA on ribosomes of *Escherichia coli* is dependent on the presence of elongation factor Tu, SmpB protein, and ribosomal protein S1. Additional proteins RNAase R, YfbG and phosphoribosyl-diphosphate synthase were co-purified with the tmRNA–SmpB complex (Karzai and Sauer 2001). Our preliminary data (Palečková *et al.* 2006) indicate that association of proteins to the tmRNA of *S. aureofaciens* is growth-phase dependent. Polyribonucleotide-nucleotidyltransferase (PNPase) was identified among proteins associated to tmRNA. The enzyme is 3′,5′-exoribonuclease catalyzing phosphorolytic degradation of RNA. In *S. antibioticus*, the expression of *pnp* gene encoding PNPase is regulated by functional promoter in the intergenic region (Bralley and Jones 2004) with structure similar to the sigma factor (σ^S), which is expressed during growth cessation or starvation. This mechanism could be involved in the regulation of PNPase expression and consequently in changes of the tmRNA level.

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