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The *in vivo* Expression of *Streptomyces rimosus* tRNA Genes

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Summary

The expression of seven tRNA genes from *Streptomyces rimosus*, cloned on bifunctional plasmids, has been studied in a homologous (*S. rimosus*) and a heterologous (*Escherichia coli*) system. Analyzed genes included cluster of genes containing two $tRNA^{Gln}$ and three $tRNA^{Glu}$ genes and two independent $tRNA^{fMet}$ genes. Northern hybridization analysis showed that all tRNA genes on plasmids are transcribed and processed in the homologous system. In the *E. coli* system only the cluster of Gln-Glu tRNA genes is properly expressed. From the deletion experiments it can be concluded that in both species all five genes in the cluster are cotranscribed from the same promoter, located 140–65 bp upstream from the first gene. A sequence TTGGAC-17-TAATGT resembling to *Streptomyces-E. coli* (SEP) promoter is located in this region. Similar sequence TTGCGC-18-TAGACT was also found 13 bp upstream from the $tRNA^{fMet1}$ gene. However, this gene is not properly expressed in *E. coli*. Putative promoter of the $tRNA^{fMet2}$ gene could not be easily identified by sequence homology in relation to two other presumptive promoters of tRNA genes. Streptomycetes promoters show huge sequence heterogeneity and two $tRNA^{fMet}$ genes obviously have very different promoters.

Key words: Streptomyces rimosus, expression, promoter, tRNA genes

Introduction

Streptomyces are Gram-positive mycelial soil bacteria, anticipated to be evolutionary highly developed prokaryotes. Upstream regions of *Streptomyces* genes show high degree of sequence divergence *(1)*. The predominant class of sequences that initiate transcription in *Streptomyces* lacks any homology with *E. coli* and *B. subtilis* promoter regions. Unlike the A+T-rich promoter regions of *E. coli* and *B. subtilis (2)*, the base composition of such *Streptomyces* DNA fragments reflects the high G+C content of the *Streptomyces* DNA *(1)*. Only about 10 % of *Streptomyces* promoters can function as transcription initiation signals in both *Streptomyces* and *E. coli*. This class of promoters denoted SEPs (*Streptomyces-E. coli* type promoters) are typically associated with housekeeping genes *(3)*. They contain all the elements characteristic of *E. coli* σ^{70} promoters, *i.e.* a –35 and a –10 region and a spacing of 16–18 bp between those regions *(4)*. Several reported promoters have only –10 region and show complete lack of homology with prokaryotic consensus in –35 region *(5–7)*.

Regulatory sequences involved in gene expression in species *Streptomyces rimosus (8–12)* are generally poorly investigated. The only well studied promoters in *Streptomyces rimosus* are promoters of *otcC* and *otcX*

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genes, members of the cluster of oxytetracycline producing genes *(12)*. Both promoters contain sequences that are similar to the consensus sequences for the –10 and –35 region of the major class of promoters in eubacteria and to the *E. coli* like promoters in Streptomycetes *(13)*.

In *E. coli* the expression of tRNA and rRNA genes plays a dominant role in the regulation of growth *(14)*. There are only few reports about the mode of expression of tRNA genes in *Streptomyces (15–17)*. Nuclease S1 mapping revealed that *GlyUβ* gene from *S. lividans* is transcribed from two promoters located 70 and over 180 bp upstream from the start of the $tRNA^{Gly}$ gene *(15)*. The transcription start point of *bldA* gene ($tRNA^{Leu}_{UUA)}$ from *S. coelicolor* was identified 69 to 70 nucleotides upstream of the 5' end of the mature tRNA and 11 nucleotides downstream of the *E. coli*-like promoter sequence *(16)*.

We analyzed regulatory sequences of two $tRNA^{fMet}$ genes *(8)* and of a cluster of five genes containing two $tRNA^{Gln}$ and three $tRNA^{Glu}$ genes *(9)* from *S. rimosus* that were cloned into bifunctional plasmids and introduced in *S. rimosus* and *E. coli*, respectively. To roughly estimate localization of the promoter of Gln-Glu tRNA gene cluster we performed deletion experiments in combination with Northern hybridization analysis of total tRNAs extracted from cells transformed with different plasmid constructs.

Materials and Methods

Bacterial strains and plasmids

E. coli strains DH1 *(18)* and GM119 *(19)* and S. *rimosus* R6-554 *(9)* were used as hosts for recombinant plasmids. *E. coli* strains were maintained and transformed as described by Maniatis *et al. (20)* and *S. rimosus* R6-554 *(9)* as described by Hopwood *et al. (21)*. *S. rimosus* protoplasts, kindly provided by Dr. J. Pigac, were prepared and regenerated according to Hopwood *et al. (21)*.

Bifunctional *E.coli-Streptomyces* plasmids pZG5 and pZG6 *(22)* and pBluescribe M13 (Stratagene) served as cloning vectors for tRNA genes.

Standard DNA procedures and manipulations

Digestion of DNA with restriction enzymes, ligation of DNA fragments, gel electrophoresis of DNA and isolation of plasmid DNA from *E. coli* were performed as described in Maniatis *et al. (20)*. Isolation of plasmid DNA from *S. rimosus* was performed as described by Hopwood *et al. (21)*.

Description of recombinant plasmids containing tRNA genes

For the purpose of this work several new recombinant plasmids were constructed. All tRNA genes from *S. rimosus*, originally cloned in *E. coli* vector pBluescribe M13, were recloned in bifunctional plasmids pZG5 and/or pZG6. Construction of recombinant plasmids, their selection and analysis were done in *E. coli* DH1 cells. Prior to the transformation of *S. rimosus* protoplasts, all plasmids were reamplified in *E. coli* GM 119 strain, deficient in DNA methylation. Only unmethylated plasmid DNA can be efficiently used for transformation of *S. rimosus (22)*.

Cluster of $tRNA^{Gln}$ and $tRNA^{Glu}$ genes *(9)*, originally cloned as 1100 bp long *Sau*IIIA/*Sma*I DNA fragment into pBluescribe M13 (Fig. 1B), contains in the 5' region the sequence TTGGAC-17-TAATGT. This sequence, located 111–83 bp upstream from the first gene in the cluster (Fig. 1A), represents a potential SEP promoter. For the purpose of this work we constructed recombinant plasmids with shorter DNA inserts, using restriction sites present in the original DNA fragment: *Sau*IIIA, *Alu*I, *Sma*I and *Bgl*II (Fig. 1B). PG5 fragment carries all 5 tRNA genes and potential promoter, while fragment G5 lacks part of the 5'-region (including potential promoter) and G4 encodes only last four genes from the cluster (Fig. 1B). In PG1 fragment, last four genes from the cluster are not present. These truncated fragments were recloned from pBluescribe M13 into pZG5 and/or pZG6 as *Eco*RI/*Xba*I fragments.

DNA fragments denoted FM1 and FM2 in this work (Fig. 2B) were recloned as *Eco*RI/*Xba*I fragments from pBluescribe M13 into pZG5 and pZG6, respectively. Fragment FM1 carries $tRNA^{fMet1}$ gene *(8)* and includes only 123 bp of DNA in the 5'-flanking region. The sequence TTGCGC-18-TAGACT (Fig. 2A), resembling to *Streptomyces-E. coli* promoter, is located in this region. Fragment FM2, encoding $tRNA^{fMet2}$ *(8)*, carries 500 bp of DNA in front of the tRNA gene (Fig. 2B). Only the potential –10 region (TACCGT) is located close to the coding region (Fig. 2A), while *E. coli*-like –35 region does not exist. Potential promoter regions of two $tRNA^{fMet}$ genes are very different.

Isolation of low molecular weight RNA

For the isolation of low molecular weight RNA (LMW RNA) we adapted the method described by Holley *(23)*. Bacterial cells were washed twice by centrifugation at 5000 × g in TES buffer *(21)*. *S. rimosus* mycelium (1 g wet weight) or 0.1 g of *E. coli* cells was mixed with 1 mL of phenol, at pH=5.2 and 2 mL of H_2O at room temperature for 2 h, and LMW RNAs were extracted by intensive stirring. Nucleic acids were precipitated from aqueous phase in 70 % of ethanol and 0.2 % potassium acetate and centrifugation at 11.000 × g for 10 min. Precipitate was washed twice with 70 % ethanol. Air dried precipitate was briefly dissolved in TE buffer, pH=8.0. LMW RNAs dissolve quickly. The undissolved fraction was discarded. In this way, 0.5 mg RNA was obtained per 1 g of mycelium and the isolated RNA predominantly consisted of tRNAs.

Northern blotting and hybridization

For Northern blotting experiments RNA (5–15 μg per lane) was separated by electrophoresis in denaturing 10 % polyacrylamide gels in the presence of 8 M of urea. Electrophoresis was performed at 25 V/cm in 1 × TBE buffer *(20)*, for approximately 2 h. Gels were stained with ethidium bromide (0.5 μg/mL) in 1 × TBE buffer for 10 min and then photographed.

After electrophoresis the RNA was transferred on an Amersham Hybond N membrane by electroblotting at 4 °C in TAE buffer *(20)* at 0.2 A for 90 min and immobilized by baking the membrane for 2 h at 80 °C.

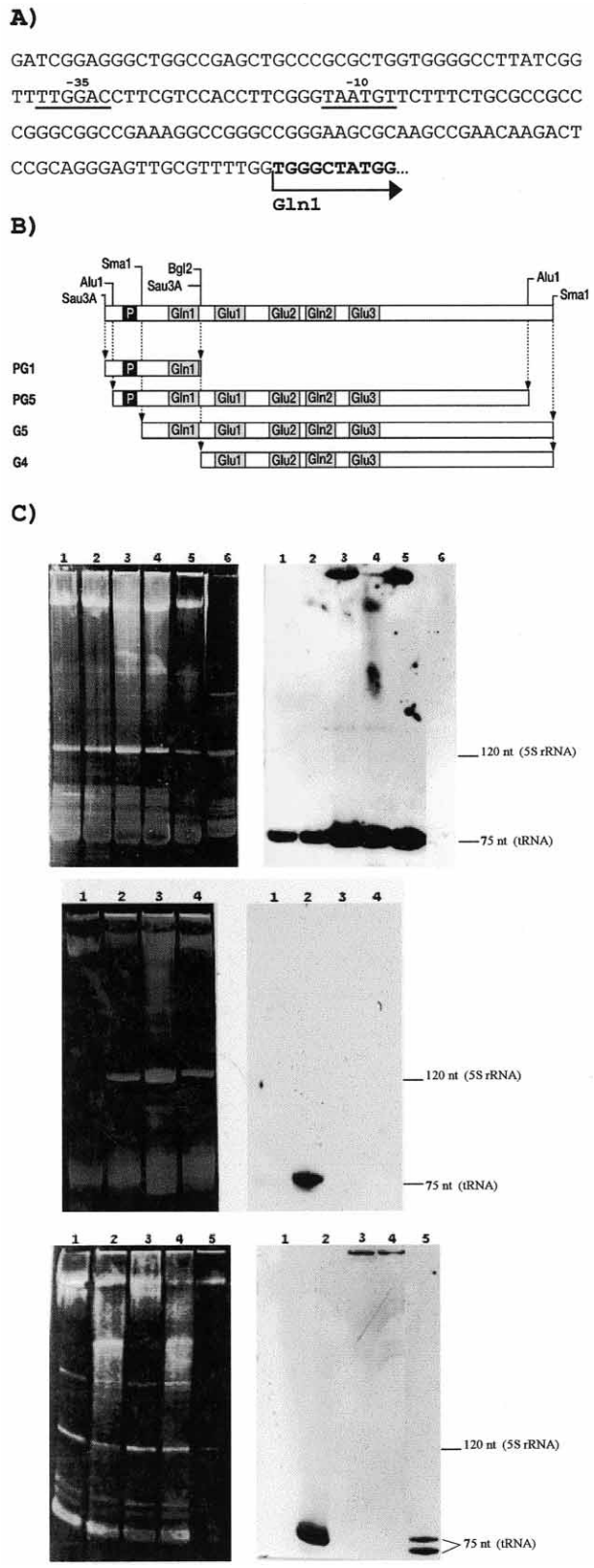

Fig. 1. A) DNA sequence upstream from the cluster of $tRNA^{Gln}$ and $tRNA^{Glu}$ genes including first ten nucleotides of the coding region; the –10 and –35 regions of potential promoter are underlined

B) Schematic representation of the DNA fragment with $tRNA^{Gln}$ and $tRNA^{Glu}$ genes and its four deletion derivatives named PG1, PG5, G5 and G4; coding regions, potential promoter and restriction sites used for subcloning are indicated

C) Top of the panel: Northern hybridization analysis of $tRNA^{Gln1}$ gene expression from PG1 in *S. rimosus*;
Left) Electrophoresis in 10 % denaturing polyacrylamide gel of LMW RNA isolated from *S. rimosus* (~15 μg) transformed with pZG6 (lanes 1 and 2), pZG5-PG1 (lanes 3 and 4) and pPZG6-PG1 (lane 5); in lane 6 there is LMW RNA isolated from *E. coli* (~15 μg) transformed with pZG6. RNA in lanes 1, 3 and 5 was isolated after 20 and that in lanes 2 and 4 after 40 h of bacterial cultivation
Right) The autoradiograph obtained after RNA transfer to membrane and hybridization with radiolabeled PG1 fragment
Middle of the panel: Northern hybridization analysis of $tRNA^{Gln}$ and $tRNA^{Glu}$ genes expression in *S. rimosus*
Left) Electrophoresis in 10 % denaturing polyacrylamide gel of LMW RNA isolated after 40 h of cultivation from *S. rimosus* (~5 μg) transformed with: pZG6 (1), pPZG6-PG5 (2), pPZG6-G5 (3) and pPZG6-G4 (4)
Right) The autoradiograph obtained after RNA transfer to membrane and hybridization with radiolabeled PG5 fragment
Bottom of the panel: Northern hybridization analysis of $tRNA^{Gln}$ and $tRNA^{Glu}$ genes expression in *E. coli*
Left) Electrophoresis in 10 % denaturing polyacrylamide gel of LMW RNA isolated from *E. coli* (~10 μg) transformed with: pZG6 (1), pPZG6-PG5 (2), pPZG6-G5 (3) and pPZG6-G4 (4); in lane 5 is LMW RNA from *S. rimosus* (~1 μg) transformed with pZG6
Right) The autoradiograph obtained after RNA transfer to membrane and hybridization with radiolabeled PG5 fragment
Although both, $tRNA^{Gln}$ and $tRNA^{Glu}$ molecules are 75 nucleotides in length, two signals can be observed in some lanes after hybridization with PG5 probe. Differences in mobility are due to difference in charges of mature tRNAs
Differences between three autoradiographs in signal intensities relative to the amount of tRNA loaded are due to difference in exposition time

For the preparation of hybridization probes, DNA fragments encoding tRNA genes were radiolabeled by random priming method *(20)*, using 25 ng of DNA and 25 μCi of [α-^{32}P]dATP (111 TBq/mmol, NEN Du Pont).

Membranes were first soaked in 6 × SSPE buffer *(20)* and then prehybridized at 30 °C for 3 h in 50 % formamide, 6 × SSPE, 0.5 % SDS, 5 × Denhardt's solution *(20)* and 100 μg/mL of denaturated, sonicated salmon sperm DNA. Hybridization was also at 30 °C for 20 h in 50 % formamide, 5 × SSPE, 0.1 % SDS, 2 × Denhardt's solution and DNA probe at a concentration of approximately 2.5 ng/mL. After hybridization, membranes were washed twice at 30 °C in 2 × SSC and 0.1 % SDS for 10 min.

Autoradiographs were obtained using Kodak XAR-5 films and Du Pont intensifier screens at –70 °C, after 24 – 48 h of exposure.

Results and Discussion

In this experimental approach to the expression analysis of the *S. rimosus* tRNA genes of interest direct Northern hybridization method was used. Cloning of 5'-flanking regions of the interest, together with their

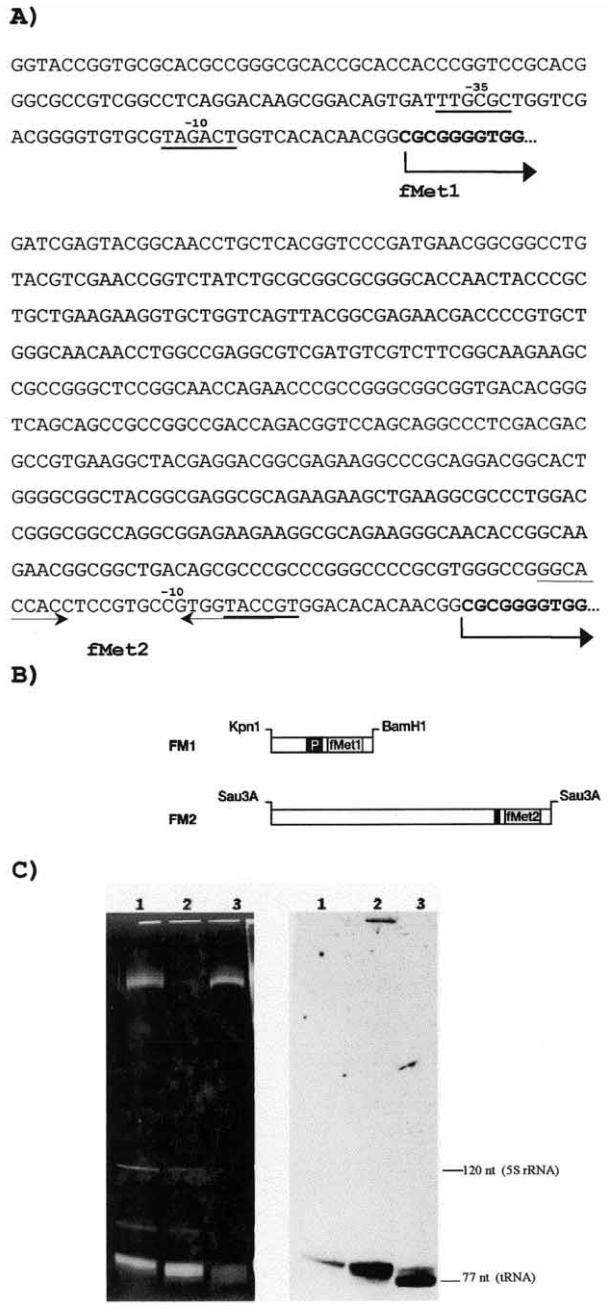

Fig. 2. A) DNA sequences upstream from the $tRNA^{fMet1}$ and $tRNA^{fMet2}$ genes including first ten nucleotides of coding regions; the –10 and –35 regions of potential promoters are underlined. Inverted repeat sequence in $tRNA^{fMet2}$ gene region is indicated by facing arrows
B) Schematic representation of FM1 and FM2 DNA fragments with $tRNA^{fMet1}$ and $tRNA^{fMet2}$ genes, respectively; coding regions, potential promoters and restriction sites used in cloning are indicated
C) Northern hybridization analysis of $tRNA^{fMet}$ genes expression in *S. rimosus*
Left) Electrophoresis in 10 % denaturing polyacrilamide gel of LMW RNA isolated from *S. rimosus* (~5 μg) transformed with: pZG6 (lane 1), pZG5-FM1 (lane 2), and pZG6-FM2 (lane 3)
Right) The autoradiograph obtained after RNA transfer to membrane and hybridization with radiolabeled FM1 fragment

corresponding tRNA genes, enabled us to detect real gene products. The use of shuttle vectors made possible the transformation of two different prokaryotic hosts with the same plasmid constructs. We predicted a higher relative abundance of certain tRNAs in transformed cells, which contain extrachromosomal copies of corresponding, transcriptionally active, tRNA genes. LMW RNA was isolated from *S. rimosus* and *E. coli* cells transformed with bifunctional plasmids containing different DNA fragments encoding *S. rimosus* tRNA genes. Some of the cloned DNA fragments carried and some did not carry potential promoters. Endogenous tRNA, obtained from cells transformed with the sole vector, served as a negative control. Increase in the abundance of tRNAs of interest in different transformants was used as the proof for presence of the active promoter on plasmid construct.

Northern hybridization analysis of $tRNA^{Gln}$ and $tRNA^{Glu}$ gene expression in homologous and heterologous system

Results of this analysis are shown on Fig. 1C. All results clearly indicate that potential SEP promoter, located at position –111 to –83 from the first tRNA gene (Fig. 1A), is indeed the promoter of all genes in the cluster in both systems (*S. rimosus* and *E. coli*).

To exclude the possibility of cotranscription of tRNA genes from the promoter of thiostrepton gene, present on pZG5 and pZG6 vectors, PG1 fragment (Fig. 1B) was cloned in both plasmids. In pZG5 vector, the possibility of cotranscription is excluded and $tRNA^{Gln1}$ gene on PG1 fragment can be transcribed only if its own promoter is present in 159 bp of DNA 5' from the tRNA gene. Hybridization signals (Fig. 1C, top of the panel), that are the results of $tRNA^{Gln1}$ gene expression from PG1 fragment cloned on pZG5 (lanes 3 and 4) and pZG6 (lane 5), are mutually the same and much stronger than signals from untransformed cell, which contain only basic amount of $tRNA^{Gln1}$ (lanes 1 and 2). It can be therefore concluded that in both plasmids transcription started from the promoter of tRNA gene(s), located within the boundaries of PG1 fragment. Additionaly, faint hybridization signal, visible in Fig. 1C (top of the panel, lanes 2, 3 and 4) comes from approximately 150 nucleotides long RNA, which could be the unprocessed pre-tRNA. If the promoter is really located 111–83 bp upstream from the first gene in cluster, as predicted from sequence analysis, then precursor tRNA with mature 3'-end is indeed 150 nucleotides in length.

This hybridization analysis did not show differences in relative abundance of $tRNA^{Gln}$ molecules in *S. rimosus* cell cultures that were grown 20 or 40 h (Fig. 1C, top of the panel, right), indicating that promoter under observation is equally active in these two different phases of the cell cycle.

To answer the question whether the transcription really starts from the potential promoter located 111-83 bp upstream from the first gene in the cluster, expression of tRNA genes in the cluster was examined in *S. rimosus* cells transformed with pZG6 carrying PG5, G5 and G4 fragments (see Materials and Methods and Fig. 1B). Results are shown in Fig. 1C, middle of the panel. Strong

hybridization signal (increased amount of $tRNA^{Gln}$ and $tRNA^{Glu}$) appeared only in cells transformed with pZG6--PG5 (lane 2), the only plasmid that contains the potential promoter region. Genes on pZG6-G5 and pZG6-G4 are transcriptionally inactive in *S. rimosus* (lanes 3 and 4). These results also excluded the possibility that transcription could be reinitiated from a (second) promoter located in intergenic regions between $tRNA^{Gln}$ and $tRNA^{Glu}$ genes. From these results, as well as results obtained with PG1 fragment ($tRNA^{Gln1}$ transcription), conclusion emerges that all $tRNA^{Gln}$ and $tRNA^{Glu}$ genes in the cluster make one unit of transcription. All tRNA genes in cluster are cotranscribed from the common promoter that lies between *Alu*I and *Sma*I restriction sites, *i.e.* between positions –140 and –65 upstream from the first tRNA gene (Fig. 1A and 1B). In this region, at positions –111 to –83, is located a sequence (TTGGAC-17-TAATGT) with high homology to SEP promoter consensus sequence. The same –35 and –10 boxes (with exception of one nucleotide) are also present in front of the Gln-Glu cluster from *S. lividans* between positions –116 and –88 *(15)*.

Transcription of $tRNA^{Gln}$ and $tRNA^{Glu}$ gene cluster was examined from the same plasmid constructs also in heterologous *E. coli* system. Results were the same as in *S. rimosus*: tRNA genes in the cluster are transcribed efficiently only from pZG6-PG5 (Fig. 1C, bottom of the panel, lane 2). This result shows that, as expected, *E. coli* RNA-polymerase specifically recognizes this SEP promoter from *S. rimosus.* pZG plasmids have much higher copy number in *E. coli* than in *S. rimosus*. However, relative intensity of signals is lower in *E. coli* than in *S. rimosus*, indicating that expression from this SEP promoter is much more efficient in *S. rimosus* than in *E. coli.*

Northern hybridization analysis of $tRNA^{fMet}$ genes expression in homologous and heterologous system

Results of these experiments are shown in Fig. 2C. Difference in intensity between hybridization signals originating from tRNAs isolated from *S. rimosus* cells transformed with pZG5-FM1 (lane 2) and pZG6-FM2 (lane 3) on one side and those from *S. rimosus* endogenous tRNA on the other side (lane 1) clearly shows that both $tRNA^{fMet}$ genes are transcriptionally active. The promoter of $tRNA^{fMet}$ genes is therefore located on FM1 and on FM2 DNA fragments.

In the heterologous system, transcription of *S. rimosus* $tRNA^{fMet}$ genes from pZG5-FM1 and pZG6-FM2 recombinant plasmids could not be detected (data not shown). tRNAs isolated from *E. coli* cells transformed with pZG5-FM1 or pZG6-FM2 displayed hybridization signals of the same intensity as tRNAs from nontransformed cells. Negative result of heterologous expression of $tRNA^{fMet1}$ gene is somewhat unexpected, because the most probable promoter on FM1 fragment TTGCGC-18--TAGACT (Fig. 2A) differs in only one base pair from the *Streptomyces* pIJ101A SEP promoter that is active in *E. coli (24)*.

Coding regions of two $tRNA^{fMet}$ genes differ in two base pairs, while there is a complete lack of homology in regions upstream of these two genes (Fig. 2A). A characteristic SEP-type promoter, recognized in *Streptomyces* by sigma factor of the major class that is analogue with σ^{70} of *E. coli*, is located in 5´-flanking region of $tRNA^{fMet1}$. Presumptive promoter of $tRNA^{fMet2}$ gene shows homology with this consensus only in –10 region: (TACCGT) (Fig. 2A). Such *Streptomyces* promoters, homologous to *E. coli* consensus only in –10 region are predicted to be also recognized by one of the principal sigma factors, that are expressed in vegetative phase of *Streptomyces* life cycle *(25)*. Putative –10 region of $tRNA^{fMet2}$ gene overlaps with one of the elements of the inverted repeat (Fig. 2A), which could possibly play a role in the expression of this promoter *(26)*. Detailed inspection of the sequences upstream from the $tRNA^{fMet2}$ gene did not reveal the existence of any other potential promoter. However, we can not exclude the possibility that very unusual type of promoter on FM2 fragment is responsible for the expression of this tRNA gene. More detailed experiments should be performed (*i.e.* primer extension) to identify exactly the promoter of $tRNA^{fMet2}$ gene. In our experiments, tRNAs were isolated from liquid cultures in which expression of genes for secondary metabolism cannot occur. Both genes are active in vegetative phase. However, their expression is most probably achieved by different sigma factors and in different ways. Two $tRNA^{fMet}$ differ in one significant base pair *(8)* and potentially could have different functions in *S. rimosus*.

Conclusions

Cluster of $tRNA^{Gln}$ and $tRNA^{Glu}$ genes from *S. rimosus* is transcriptionally active in a homologous and in a heterologous system (*E. coli)*. All $tRNA^{Gln}$ and $tRNA^{Glu}$ genes in cluster are cotranscribed from one common promoter and they all make one unit of transcription. Promoter activity in a homologous, as well as in a heterologous system, is bound to a DNA segment that is 140–65 bp upstream from the first gene in the cluster. In this region, (position –111 to –83) SEP promoter TTGGAC-17-TAATGT, which can be recognized by RNA-polymerases from both bacteria, is located.

Two $tRNA^{fMet}$ genes from *S. rimosus* are transcriptionally active only in homologous system. They have significantly different promoters which do not function in *E. coli*.

Acknowledgments

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In vivo ekspresija tRNA gena bakterije *Streptomyces rimosus*

Sažetak

Proučena je ekspresija sedam tRNA gena bakterije *Streptomyces rimosus*, kloniranih na bifunkcionalnim plazmidima u homolognom (*S. rimosus*) i heterolognom (*Escherichia coli*) sustavu. Analizirana su dva neovisna gena za $tRNA^{fMet}$ te skupina gena koju čine dva gena za $tRNA^{Gln}$ i tri gena za $tRNA^{Glu}$. »Northern« hibridizacijska analiza pokazala je da se svi geni za tRNA na plazmidima transkribiraju i procesiraju u homolognom sustavu. U sustavu *E. coli* pravilno se eksprimira samo skupina gena za $tRNA^{Gln}$ i $tRNA^{Glu}$. Na osnovi delecijske analize zaključeno je da se u obje vrste svih pet gena iz skupine transkribira sa zajedničkog promotora, smještenog 140–65 parova baza uzvodno od prvoga gena. U tom području smješten je slijed nukleotida TTGGAC-17-TAATGT, visokog stupnja homologije sa *Streptomyces-E. coli* (SEP) promotorom. Sličan slijed: TTGCGC-18-TAGACT također je nađen 13 parova baza uzvodno od gena za $tRNA^{fMet1}$. Međutim, ovaj se gen ne eksprimira pravilno u *E. coli*. Potencijalni promotor gena za $tRNA^{fMet2}$ nije bilo moguće ustanoviti na osnovi homologije s preostala dva predvidiva promotora gena za tRNA. Promotori gena u streptomiceta pokazuju izrazito veliku heterogenost sekvencija i očito je da dva gena za $tRNA^{fMet}$ imaju sasvim različite promotore.