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5' Untranslated Region of the *Pseudomonas putida* WCS358 Stationary Phase Sigma Factor *rpoS* mRNA is Involved in RpoS Translational Regulation

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The σ^S subunit of RNA polymerase is a central regulator which governs the expression of a host of stationary phase-induced and osmotically regulated genes in Gram-negative bacteria. The *Pseudomonas putida rpoS* gene is transcribed as a monocistronic *rpoS* mRNA with a 368 nucleotide-long 5' untranslated region (5' UTR). In this study, we investigate the posttranscriptional control of RpoS synthesis using *rpoS-lacZ* transcriptional and translational fusions consisting of the native promoter and deletions of 5' UTR or insertion into UTR. The differing activity of constructed translational fusions strongly indicated that the 5' UTR is involved in the translational regulation of RpoS expression in the stationary phase. The results obtained herein demonstrated that the structure of UTR performs an important function in the translational regulation of the *rpoS* gene.

***Keywords*: *Pseudomonas*, *rpoS*, translational regulation**

Bacteria often encounter constantly changing nutrient availability and exposure to a variety of physical stresses, including osmotic stress, oxidative stress, and temperature shock. These environmental conditions result in a reduction in or cessation of growth, referred to as the stationary phase, which results in a major switch in gene expression which allows the cells to cope. The stationary phase alternative sigma factor, σ^S (also called σ^{38} and RpoS), initially described in *Escherichia coli*, has been identified in many non-enteric bacteria belonging to the γ-subdivision of Proteobacteria. The levels of σ^S are carefully controlled, and increase dramatically at the onset of the stationary phase, increasing its ability to compete with other available σ subunits for core RNA polymerase (Jishage *et al*., 1996). The regulation of *rpoS* expression in *E. coli* takes place at the level of transcription, translation, and protein stability, all coordinates by the response to several stress signals (Hengge-Aronis, 2002). The *rpoS* transcript of *E. coli* originates in the *nlpD* gene located upstream, which generates a monocistronic mRNA with a long 5' untranslated region (5' UTR) consisting of 567 bp; the *rpoS* mRNA secondary structure, coupled with *trans*-acting factors and small regulatory RNAs, controls translational initiation under differing environmental conditions (Jishage *et al*., 1996). The regulation of *rpoS* has been addressed recently in the fluorescent pseudomonads, where transcriptional regulation apparently performs the principal role (Kojic *et al*., 2002; Venturi, 2003). As in *E. coli*, in *P. putida* and *P. aeruginosa* the *rpoS* transcript originates within the upstream *nlpD* gene, generating monocistronic mRNA with a 5' UTR consisting of 373 bp (Hengge-Aronis, 2002). It was revealed in a recent study that the S1 ribosomal protein binds preferentially to the 5' UTR of *P. aeruginosa rpoS* mRNA in the logarithmic growth phase, and does not in the stationary phase, and also represses the translation of *rpoS* mRNA (Sevo *et al.*, 2004). In order to determine the role of the *rpoS* 5' UTR in the translational regulation of RpoS expression in *P. putida* WCS358 we conducted mutation, deletion, and insertion analyses of this untranslated region.

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Materials and Methods

Bacterial strains, plasmids, media, and chemicals

The strains employed in this study included *E. coli* DH5α, *E. coli* pRK2013, and *P. putida* WCS358. *E. coli* and *P. putida* strains were grown in LB medium at 37℃ and 30℃, respectively. The following antibiotic concentrations were utilized: ampicillin, 100 μg/ml (*E. coli* and *P. putida*); kanamycin, 100 μg/ml (*E. coli*); chloramphenicol, 35 μg/ml (*E. coli*) and 500 μg/ml (*P. putida*); tetracycline, 15 μg/ml (*E. coli*) and 50 μg/ml (*P. putida*). The plasmids utilized in this study are listed in Table 1.

Recombinant DNA techniques

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with the Klenow fragment of DNA polymerase, and transformation of *E. coli* were conducted as previously described (Sambrook *et al*., 1989). Analytical amounts of plasmids were isolated as previously described (Birnboim, 1983), whereas preparative amounts were purified using QIAGEN columns. The primers utilized for PCR analyses are listed in Table 1. *Taq* polymerase and buffers were purchased from Perkin-Elmer Cetus, and 50 μl reactions were conducted on an Eppendorf Mastercycler personal.

Table 1. Plasmids and primers used in this study (for details see 'Materials and Methods')

Plasmid/Primer	Relevant characteristics/Primer sequence	Origin/Reference
pBluescript SK	Amp^r, ColE1 replicon	Stratagene
pBluescript KS	Amp^r, ColE1 replicon	Stratagene
pMP220	Promoter cloning vector, IncP1, Tc^r	Spaink *et al.* (1987)
pMP77	Promoter cloning vector, IncQ, Cm^r	Spaink *et al.* (1987)
pH3.5*lacZ*2	pBluescript-*rpoS* genome clone with *lacZ* cloned in *Aat*II restriction site of *rpoS* gene	Bertani *et al.* (2003)
pBSΔSS	pBluescript SK with deletion of *lacZ2* gene (*Ssp*I-*Sal*I) region	This study
pBSPE	*Pst*I-*Eco*RI promoter region of *rpoS* gene cloned into pBluescript KS	This study
pBSK	*Pst*I-*Hin*dIII fragment of pH3.5*lacZ2* cloned into pBSΔSS	This study
pMP77FK	pBSK digested with *Hin*dIII and cloned into pMP77	This study
pMP77F3	pBSF3 digested with *Hin*dIII and cloned into pMP77	This study
pMP77F4	pBSF4 digested with *Hin*dIII and cloned into pMP77	This study
pMP77F5	pBSF5 digested with *Hin*dIII and cloned into pMP77	This study
pMP77F6	pBSF6 digested with *Hin*dIII and cloned into pMP77	This study
pMP77F7	pBSF7 digested with *Hin*dIII and cloned into pMP77	This study
pMP77FΔEI	pBSFΔEI digested with *Hin*dIII and cloned into pMP77	This study
pMP77FΔE47III	pBSFΔE47III digested with *Hin*dIII and cloned into pMP77	This study
pMP77FINEI	pBSFINEI digested with *Hin*dIII and cloned into pMP77	This study
pMP220FK	*Bam*HI-*Kpn*I fragment from pBSFKT cloned into pMP220	This study
pMP220F3	*Bam*HI-*Kpn*I fragment from pBSF3T cloned into pMP220	This study
pMP220F4	*Bam*HI-*Kpn*I fragment from pBSF4T cloned into pMP220	This study
pMP220F5	*Bam*HI-*Kpn*I fragment from pBSF5T cloned into pMP220	This study
pMP220F6	*Bam*HI-*Kpn*I fragment from pBSF6T cloned into pMP220	This study
pMP220F7	*Bam*HI-*Kpn*I fragment from pBSF7T cloned into pMP220	This study
pMP220FΔEI	*Bam*HI-*Kpn*I fragment from pBSFΔEIT cloned into pMP220	This study
pMP220FE47III	*Bam*HI-*Kpn*I fragment from pBSFΔE47IIIT cloned into pMP220	This study
pMP220FINEI	*Bam*HI-*Kpn*I fragment from pBSFINEIT cloned into pMP220	This study
EcoRV1F	GTCTGATATCGAACTCAGC	
EcoRV1R	GTTCGATATCAGACTCGAGCGG	
EcoRI1F	GAGCTGAATTCATCAAGCACAGCG	
EcoRI1R	GTGCTTGATGAATTCAGCTCGGCG	
EcoRI2F	GGCTTTTGAATTCGGGAGGGGCAG	
EcoRI3F	ATTGCTGAATTCGTCCACGGGCAC	
EcoRI4F	TTTCGAGAATTCGCCGCCAGGGC	
EcoRI5F	CCGTTGAATTCTTGTACCCGGCC	

Amp^r, Tc^r, and Cm^r, resistant to ampicillin, tetracycline and chloramphenicol, respectively. Restriction sites are underlined in the primer sequence.

Triparental matings from *E. coli* to *Pseudomonas* were conducted with an *E. coli* (pRK2013) helper strain via a previously described method (Figurski and Helinski, 1979). The deletions of 5' UTR by exonuclease III were conducted using a Nested deletion kit (Pharmacia Biotech, USA).

Construction of translational and transcriptional fusions

Translational fusions of the *rpoS* gene were prepared using pBluescript and pMP77 plasmids (Table 1). PCR fragments of different sizes [293 bp (pBSPCR3), 234 bp (pBSPCR4), 193 bp (pBSPCR5), 149 bp (pBSPCR6), and 96 bp (pBSPCR7)] of *rpoS* 5' UTR were cloned into pBSΔSS resulting in pBSPCR3, pBSPCR4, pBSPCR5, pBSPCR6, and pBSPCR7 intermediate constructs. Upstream of the cloned PCR products, the promoter region of the *rpoS* gene (*Pst*I-*Eco*RI from pBSPE) was cloned, generating the pBSP3, pBSP4, pBSP5, pBSP6, and pBSP7 constructs. The downstream of the cloned PCR fragment of the *lacZ2* gene was cloned, in frame with the proximal 40 bp of the *rpoS* gene, (*Hin*dIII-*Xho*I fragment from pH3.5*lacZ2*). The resulting fusions, pBSF3, pBSF4, pBSF5, pBSF6, and pBSF7 were digested with *Hin*dIII and cloned into pMP77, generating the following fusions:

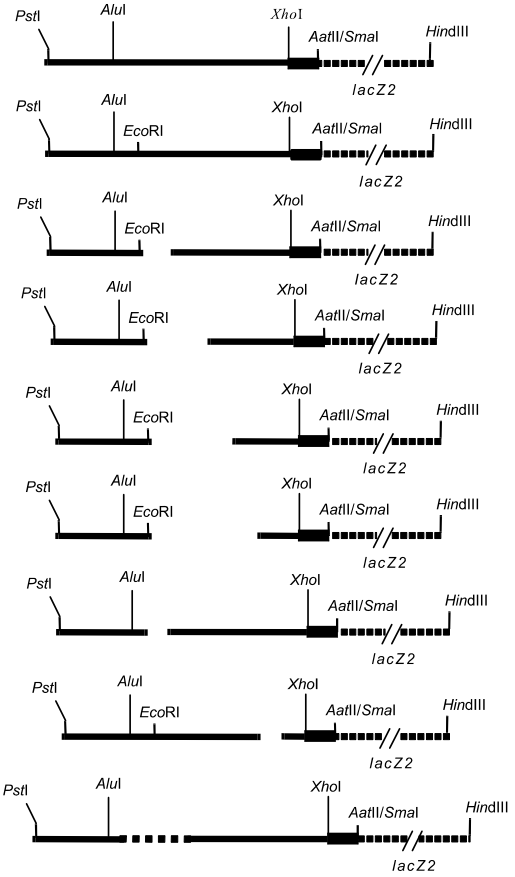
pMP77F3, pMP77F4, pMP77F5, pMP77F6, and pMP77F7. The pMP77FΔEI, pMP77FΔE47III fusions were derived from pBSP3, which was treated with exonucleaseIII in the *Eco*RI restriction site (getting pBSFΔEI with 31 bp deletion of *rpoS* 5' UTR) or in the *Eco*47III restriction site (getting pBSFΔE47III with 32 bp deletion of *rpoS* 5' UTR). The pMP77FINEI fusion was derived from pBSP3 via the insertion of a 78 bp-long DNA fragment into the *Eco*RI restriction site, generating the pBSFINEI construct. The *Hin*dIII-*Xho*I fragment from pH 3.5*lacZ2* was cloned into these intermediate constructs (pBSFΔEI, pBSFΔE47III, and pBSFINEI). Finally, the constructs were cloned into pMP77 (resulting in pMP77FΔEI, pMP77FΔE47III, and pMP77FINEI). The transcriptional *rpoS* fusions were prepared in a pMP220. *Pst*I-*Xho*I fragments from the same constructs used for the construction of translational fusions (pBSK, pBSF3, pBSF4, pBSF5, pBSF6, pBSF7, pBSFΔEI, pBSFΔE47III, pBSFINEI) were cloned separately into pBluescript, generating the pBSKT, pBSF3T, pBSF4T, pBSF5T, pBSF6T, pBSF7T, pBSFΔEIT, pBSFΔE47IIIT, and pBSFINEIT constructs. These intermediate constructs were digested with *Bam*HI-*Kpn*I restriction enzymes, and the fragments were purified and cloned into pMP220 digested with *Bgl*II-*Kpn*I, yielding pMP220FKT, pMP220F3T, pMP220F4T, pMP220F5T, pMP220F6T, pMP220F7T, pMP220FΔEIT i pMP220FΔE47IIIT, and pMP220FINEI. The translational and transcriptional fusions are listed in Table 1 (data not shown).

β-Galactosidase assays

An overnight culture (10 μl) of *P. putida* WCS358 harboring different translational/transcriptional fusions was resuspended in Z-buffer (60 mM $Na_2HPO_4{\cdot}7H_2O$, 40 mM $NaH_2PO_4{\cdot}H_2O$, 10 mM KCl, 1 mM $MgSO_4{\cdot}7H_2O$) and then permeabilized by treatment with sodium dodecyl sulfate and chloroform. Assays were conducted in Z-buffer containing 50 mM β-mercaptoethanol (Miller, 1972). Activities [changes in optical density at 420 nm (OD_{420}) per min] were normalized to the actual cell density (OD_{600}) and were consistently compared to those of the appropriate controls, which were assayed at the same time. The results presented are all from unique experiments. The experiments were conducted in triplicate. In order to eliminate readthrough activity from other promoters on pMP220, conjugants with this plasmid were utilized as a negative control.

Computer analysis

The secondary structure prediction of *rpoS* 5' UTR mRNA and fragments derived from it was conducted via Zuker group



	Activity
pMP77FK	100%
pMP77F3	77.7%
pMP77F4 (Δ50 bp)	15.4%
pMP77F5 (Δ100 bp)	35.8%
pMP77F6 (Δ150 bp)	15.5%
pMP77F7 (Δ200 bp)	36.1%
pMP77FΔEI (Δ31 bp)	23.3%
pMP77FΔE47III (Δ32 bp)	55.7%
pMP77FINEI (insertion 78 bp)	78.2%

Fig. 1. Schematic presentation of *rpoS-lacZ2* translational fusions. Each line below the top one represents a different fusion; the names and activities that refer to these fusions are listed in the columns. The relevant restriction sites utilized for a construction of fusions are indicated. 5' UTR deletions are indicated as gaps (size of each gap correspond to deletion indicated in brackets beside the name of fusion) and insertions by dashed lines.

algorithms, thermodynamics, and databases for RNA secondary structures (www.bioinfo.rpi.edu/~zukerm/rna/), (Mathews *et al.*, 1999; Zuker, 2003).

Results and Discussion

Previous studies regarding the regulation of the *rpoS* gene in *Pseudomonas* have been largely focused on the transcriptional level and have highlighted the important role performed by a TetR family regulator referred to as PsrA (Kojic *et al.*, 2002; Kojic *et al.*, 2005). A previous study regarding regulatory control at the level of translation revealed that the levels of σ^S protein were stimulated to a considerable degree at the onset of the stationary phase in wild-type *P. aeruginosa* PAO1 and also in the *psrA*::Tn*5*, which, however, harbored less RpoS protein (Bertani *et al.*, 2003; Sevo *et al.*, 2004). Taking into account the fact that there is no evidence regarding the importance of 5' UTR in terms of the regulation of RpoS expression in the stationary phase of growth, we conducted a deletion analysis of this region in order to determine its role in the regulation of the translational expression of *rpoS* gene.

Nine translational fusions, in total, were constructed (Fig. 1 and Table 1), which contained the *rpoS* promoter, *rpoS* 5' UTR (full-length, or with deletions and insertion of DNA) and the *lacZ2* gene from the mini-Tn*5* *lacZ2* transposon (De Lorenzo *et al.*, 1990). In the initial set of experiments, we assessed the β-galactosidase activities of the pMP77F3, pMP77F4, pMP77F5, pMP77F6, pMP77F7, pMP77FΔEI, pMP77FΔE47III fusions containing various deletions of the *rpoS* 5' UTR and the activity of the pMP77FINEI fusion containing the 78 bp insertion in the *Eco*RI restriction site (proximal region of 5' UTR, 60 bp downstream from the start of transcription). The pMP77FK construct, which harbors the full-length 5' UTR, was utilized as a control, and the activities of other fusions were defined as compared to its activity. The resultant activities (Fig. 1) showed that the generation of the *Eco*RI restriction site via PCR mutagenesis reduces the activity by approximately 22.3% and the deletion of that proximal region (pMP77F4) reduced activity by more than 85%. These results, coupled with the resultant activities of pMP77FΔEI and pMP77FΔE47III, which were 23.3% and 55.7%, respectively, showed that the proximal region of 5' UTR was a region which played an important role in the translational regulation of the *rpoS* gene. It was also determined that the same region is involved in interactions with the S1 protein, which is responsible for the negative regulation of the *rpoS* gene in the logarithmic growth phase (Sevo *et al.,* 2004). The results obtained in this study pointed to the proximal region of 5' UTR as the most important region involved in the posttranscriptional regulation of the *rpoS* gene, providing the optimal translation of the *rpoS* gene in *P. putida* WCS358. It is probable that signal factors that interact with the 5' UTR respond to energetic status in the cell or in the environment, as is also the case in *E. coli* (Hengge-Aronis, 2002). Surprisingly, the β-galactosidase activity of the fusion pMP77FINEI was even higher than that of pMP77F3, thereby indicating that the insertion that prolongs 5' UTR (for 78 nucleotides) exerts no influence on translational machinery. The ability of all constructed translational fusions to respond to the growth phase of *P. putida* WCS358 was also analyzed in order to assess the involvement of a variety of regions in interactions with different growth phase factors. During the growth of a *P. putida* WCS358 culture harboring different fusions, different β-galactosidase activities were observed, but the culture retained an almost identical growth phase dependence (Fig. 2, data shown only for relevant fusions), thereby indicating that several factors are involved in the translational regulation of the *rpoS* gene.

In order to eliminate the impact of deletions and insertions into the 5' UTR of the *rpoS* gene on the promoter transcriptional activity of translational fusions, the same number of transcriptional fusions were constructed. The transcriptional fusions (in pMP220 promoter probe vector) consisted of the promoter region as well as that of the 5' UTR (or its deletions and one insertion) of the *rpoS* gene; the same regions located upstream from RBS were utilized in the construction of translational fusions. No statistically significant differences between the activity values of constructed transcriptional fusions were observed. These results showed that the promoter and the changes introduced into the 5' UTR of *rpoS* gene exerted no influence on transcriptional activities.

As the results regarding the β-galactosidase activities of translational fusions of the *rpoS* gene clearly indicated that differences between the 5' UTR regions exert a significant impact on *rpoS* translation, we have conducted predictions of the mRNA secondary structures of 5' UTR fragments from those fusions (for details, see the 'Materials and Methods' section).

The resultant secondary structures (Fig. 3) showed that differences in secondary structures could explain the different activities of translational fusions. The secondary structures of the native 5' UTR from pMP77FK and 5' UTR with

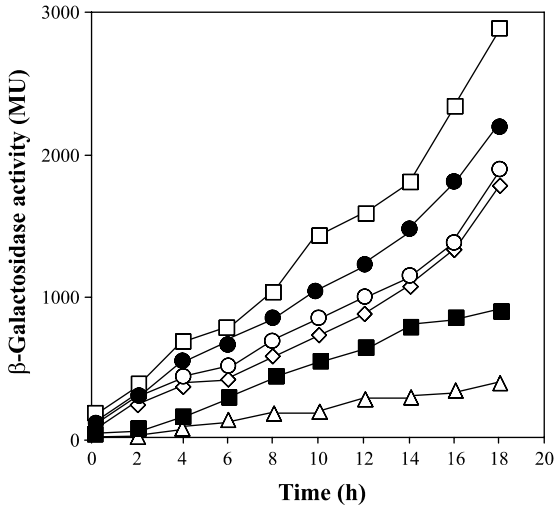


Fig. 2. The expression of *rpoS* translational fusions [pMP77FK (□), pMP77F3 (○), pMP77F5 (■), pMP77F6 (△), pMP77FΔE47III (◇), pMP77INEI (●)] in *Pseudomonas putida* WCS358. The values shown are the β-galactosidase activities expressed in Miller units (MU) per unit of time (h) during growth in LB medium. The results are expressed as the mean values of triplicate experiments.

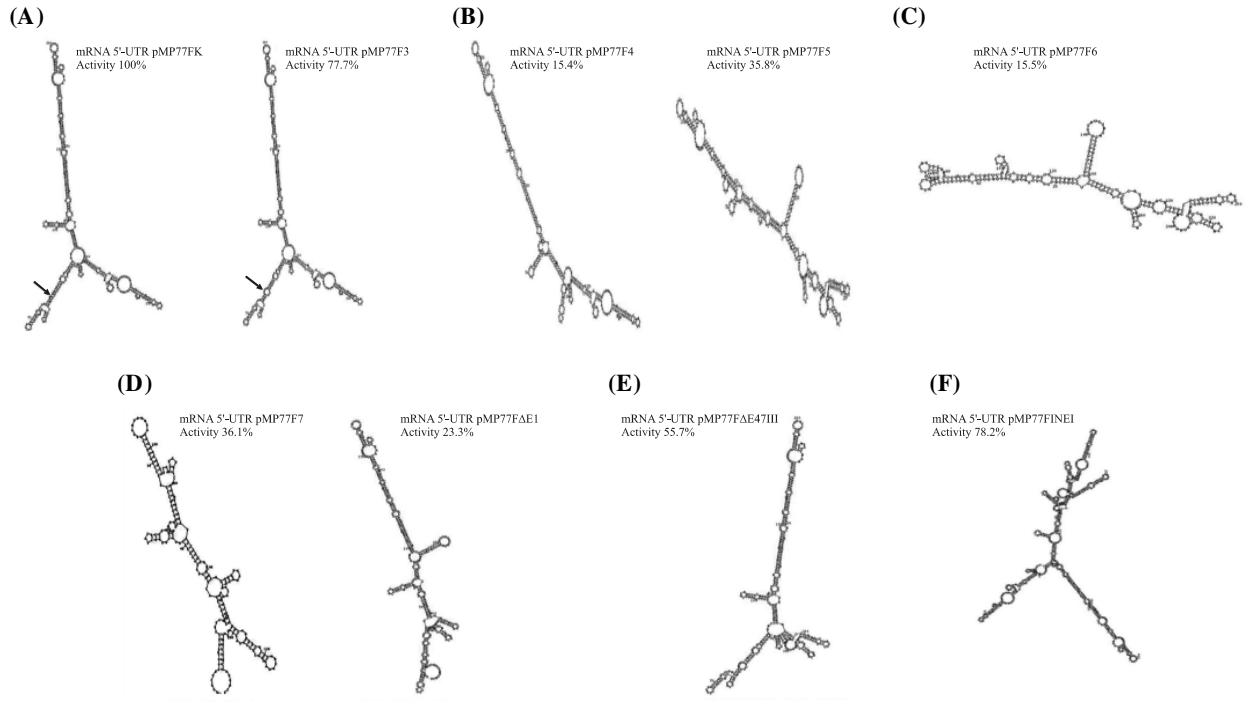


Fig. 3. Secondary structure predictions of *rpoS* 5' UTR mRNA and fragments derived from it. The arrows indicate structural differences between the secondary structures of *rpoS* 5' UTR mRNA fragments harbored by pMP77FK and pMP77F3.

generated *Eco*RI (by PCR) from pMP77F3 were almost identical, except within the region where the *Eco*RI site was created. A bubble-like structure was detected in the structure of the UTR fragment of pMP77F3 in the *Eco*RI restriction site region, but not in the structure of the UTR fragment of pMP77FK (Fig. 3, marked with arrows). This difference showed that the bubble-like region structure might have a direct effect on the efficiency of *rpoS* mRNA translation, or via interactions with other regulators (reduced activity by approximately 22.3%). In addition, the secondary structure of the mRNA of the 5' UTR fragment from pMP77FΔE47III (Fig. 3), which harbors a deleted distal 5' UTR region, strongly indicated the important role of the proximal region, as this maintains the level of activity at 55.7% as compared to the controls. Similar activities of the pMP77FINEI and pMP77F3 fusions might possibly be explained by the similarity of the secondary structures of the 5' UTR of these two fusions. The predicted secondary structures of the *rpoS* mRNA of 5' UTR were insufficient to explain the differences in the β-galactosidase activities of certain analyzed fusions (pMP77F5 and pMP77F7). Due to these results, we surmised that beside the secondary structure, some *trans*-acting factors may also be involved in the translational regulation of the *rpoS* gene. Both *cis*- and *trans*-acting factors might affect translation via the exposure or blockage of the ribosome binding site (RBS) of the *rpoS* gene, as in *E. coli* (Hengge-Aronis, 2002). Further *in vivo* and *in vitro* studies will determine the complexity and the role of 5' UTR *rpoS* mRNA, as well as the interaction between 5' UTR and other *trans*-acting factors (RNA binding proteins and small RNA) in *Pseudomonas*.

Acknowledgements

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