

# Inability of *Agrobacterium tumefaciens* Ribosomes to Translate *in vivo* mRNAs Containing Non-Shine-Dalgarno Translational Initiators

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Numerous data accumulated during the last decade have shown that the Shine-Dalgarno (SD) sequence is not a unique initiator of translation for *Escherichia coli*. Several other sequences, mostly of viral origin, have demonstrated their capability of either enhancing or initiating translation *in vivo*. A phage T7 gene 10 sequence, called "epsilon" ( $\epsilon$ ), has shown its high enhancing activity on translation in both *Escherichia coli* and *Agrobacterium tumefaciens* cells. In this study the  $\epsilon$ , together with three other nucleotide sequences derived from the 5' non-translated regions of tobacco mosaic virus (TMV), papaya mosaic virus (PMV) and clover yellow mosaic virus (CYMV) RNAs are tested for translation initiation activity in *A. tumefaciens* cells. The obtained results indicate that none of them was capable of initiating translation *in vivo* of chloramphenicol acetyltransferase (CAT) mRNA. To determine whether their inactivity was related with structural differences in the ribosomal protein S1, the *rpsA* gene (coding for S1 protein in *E. coli*) was co-expressed in *A. tumefaciens* together with the *cat* gene placed under the translational control of the above sequences. Our results showed that the *rpsA* gene product did not make any of the four viral enhancers active in *A. tumefaciens* cells. The inability of *A. tumefaciens* ribosomes to translate mRNAs devoid of SD sequences indicates for a substantial difference in the ribosome structure of the two Gram negative bacteria *E. coli* and *A. tumefaciens*.

## Introduction

It is well established that initiation of translation in *Escherichia coli* includes base pairing between a purine-rich domain at the 5' untranslated region of mRNA, called Shine-Dalgarno (SD) sequence, and a complementary sequence (anti-SD) at the 3' end of 16S rRNA (Gold, 1988). The presence of very short SD sequences or their complete absence from some natural mRNAs (Stormo, 1986; Bibb *et al.*, 1994; Wu and Janssen, 1996), however, suggests alternative mechanisms of initiation of translation (Gold, 1988; Sprengart and Porter, 1997).

A series of nucleotide sequences derived from the 5' non-translated regions of *Mycoplasma genitalium tuf* gene (Loechel *et al.*, 1991), tobacco mosaic virus RNA ( $\Omega$  sequence) (Gallie and Kado, 1989; Ivanov *et al.*, 1992), bacteriophage T7 gene

10 ( $\epsilon$  sequence) (Olins and Rangwala, 1989; Ivanov *et al.*, 1995; Golshani *et al.*, 2000a; Golshani *et al.*, 2000b), etc. have demonstrated their capability to independently initiate translation of various mRNAs in *E. coli*. In all these cases a non-conventional base pairing (rather than the canonical SD-anti-SD) has been proposed. Alternative base pairings between mRNA and 16S rRNA have also been proven by cross-linking experiments (McCarthy and Brimacombe, 1994; Baranov *et al.*, 1998). Besides the mRNA-rRNA interaction, a number of reports indicate that the ribosomal protein S1 is involved in the mRNA recognition step (van Dieijen *et al.*, 1975; van Dieijen *et al.*, 1978; Thomas and Szer, 1982; Suryanarayana and Subramanian, 1983). A model is proposed in which the S1 protein interacts with mRNA by four RNA binding domains (Subramanian, 1983; Boni *et al.*, 1991). Surprisingly, this protein is absent from the

ribosomes of some bacteria and its presence in *Agrobacterium tumefaciens* ribosomes is not proven yet.

Recently we have shown that the  $\epsilon$  sequence is an efficient enhancer of translation in both *E. coli* and *A. tumefaciens* cells (Golshani *et al.*, 2000a). We have also found that the enhancing activity correlates well with its complementarity to the corresponding ribosome binding site ( $\epsilon$ BS) located in the helical domain #17 of 16S rRNA. The latter indicates that the enhancing effect of  $\epsilon$  in both microorganisms is due to a mRNA-rRNA base pairing. In another study we have shown that the extension of complementarity of  $\epsilon$  to domain #17 from 9 nucleotides (equal to its natural length) to 16 bases is the reason for its conversion from an enhancer to an independent initiator of translation in *E. coli* cells (Golshani *et al.*, 2000b).

This study aims to investigate the capability of  $\epsilon$  and other enhancers of viral origin to independently (in the absence of SD sequences) initiate translation of CAT mRNA in *A. tumefaciens* cells.

## Materials and Methods

### Construction of expression plasmids

The translation initiation sequences (RBS) shown in Fig. 1A are synthesized on a Cyclone 7000 DNA synthesizer (Milligen). They all are flanked by *Hind III* and *Xho I* overhangs necessary for cloning into the expression plasmid pBRP<sub>1</sub>CAT (Golshani *et al.*, 1997). This plasmid was derived from the cloning vector pBR322 in which the *cat* gene (devoid of a translation initiation sequence) was placed under the transcriptional control of a strong constitutive promoter (P<sub>1</sub>). The expression cassette (consisting of a promoter, RBS sequence and a reporter gene) was then excised by *BsrBI* and sub-cloned into the *Hpa I* site of the binary plasmid pGA643 (An, 1995) as shown in Fig. 1B. The resulting shuttle vector thus obtained bore two constitutive (Tc<sup>R</sup> and Km<sup>R</sup>) and one conditional (Cm<sup>R</sup>) resistances. The latter one depended on the efficiency of *cat* gene expression, i.e. on the activity of the corresponding RBS sequence. The Km<sup>R</sup> gene was further inactivated by either *BamHI* / *Pst I* or *BamHI*

### A.

	XhoI	HindIII
SD <sub>cons</sub>	UCGAGA <b>AAGGAGG</b> UUUAGCUUAUG...	
$\epsilon_{Ecl16}$	UCGA <b>UUAAACUUU</b> AUUUACCUUAUCAGCUUAUG...	
$\epsilon_{Ail16}$	UCGA <b>UUUAUCUUCU</b> UUUACCUUAUCAGCUUAUG...	
$\epsilon_{Ail8}$	UCGA <b>UUUAUCUUCU</b> AUCAGCUUAUG...	
$\epsilon_{Ail11}$	UCGA <b>UUUAUCUUCU</b> UUGGUCAAUUCAGCUUAUG...	
$\Omega$	UCGACCUU <b>ACAAUUACU</b> AUUUACAAUUAACAAGCUUAUG...	
PMV <sub>NTR</sub>	UCGAG <sup>78nt</sup> GCACAACCCUAG <b>AUUAACGA</b> AGCUUAUG...	
CYMV <sub>NTR</sub>	UCGAG <sup>72nt</sup> AACC <b>AGCAAAG</b> AUUCGCAUUAAGCUUAUG...	
$\epsilon_{Ee/SD}$	UCGA <b>UUAAACUUU</b> AUCAAGGAGGUUUCAGCUUAUG...	
$\epsilon_{Ail/SD}$	UCGA <b>UUUAUCUUCU</b> ACAAGGAGGUUUCAGCUUAUG...	
$\Delta\epsilon/SD$	UCGAGUAUACUACUUA <b>AUGCUU</b> AUG...	

### B.

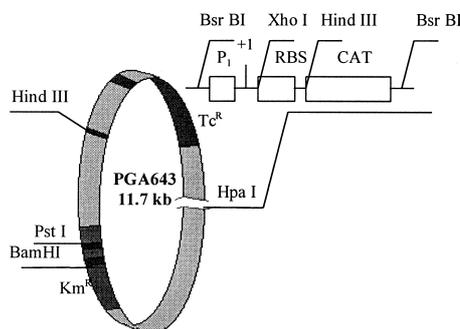


Fig. 1. Nucleotide sequences of 5' non-translated regions of CAT mRNAs (A) obtained from the expression plasmid pGA643 (B). The translation initiation sequences (RBS) in A are shown in bold letters and the initiation codons (AUG) in italics. The expression cassette in B (right) consists of a constitutive promoter (P<sub>1</sub>), cloning site for the RBS sequences and a reporter (*cat*) gene.

I/*Hind* III digestion followed by mung bean nuclease treatment and ligation.

To construct the plasmid pBIS1 (designed for expression of the *E. coli rpsA* gene in *A. tumefaciens* cells) the *rpsA* gene placed under a *lac* promoter/operator control was excised from the plasmid pJFR32 (courtesy of Dr. S. Pedersen) and re-cloned into the *Sma* I site of the binary plasmid pBII101 bearing an *A. tumefaciens* origin of replication.

All plasmids were transformed into both *E. coli* LE392 and *A. tumefaciens* PMP90 cells following standard protocols.

*A. tumefaciens* cells co-transformed with two expression plasmids were obtained as follows: bacteria carrying the plasmid pBIS1 (Km<sup>R</sup>) were further transformed with the plasmid pGA643 (Tc<sup>R</sup>) in which the Km<sup>R</sup> gene has already been destroyed as described above. The double transformants were selected by their binary (Tc<sup>R</sup>, Km<sup>R</sup>) phenotype.

#### CAT assay

*E. coli* LE392 cells transformed with the above expression plasmids were cultivated in a Luria-Bertani (LB) medium supplemented with 20 µg/ml tetracycline at 37 °C under vigorous shaking. *A. tumefaciens* PMP90 cells transformed with the same vectors were grown in LB broth supplemented with 10 µg/ml tetracycline and 25 µg/ml streptomycin at 28 °C under mild agitation. Bacterial cells (1 ml cell suspension of A<sub>590</sub> = 1.000) were harvested by centrifugation, resuspended in 1 ml TE (10 mM Tris-HCl, pH 7.0, 1 mM EDTA) buffer, disrupted by French press (1000 psi) and centrifuged. CAT activity in the clear lysates was measured spectrophotometrically as described elsewhere (Golshani *et al.*, 1997; Shaw, 1975).

#### Protein and mRNA analyses

For Western blot analysis, 10 µg of total *A. tumefaciens* protein was subjected to electrophoresis on a 12% polyacrylamide gel. Protein fractions were transferred onto nitrocellulose filters and incubated with sheep or rabbit antisera raised against purified *E. coli* protein S1 (courtesy of Drs. R. Brimacombe, M. Farwell and L. Spremulli). The filters were then treated with a secondary

(anti-rabbit or anti-sheep) IgG conjugated with alkaline phosphatase.

The content of CAT mRNA in the transformed cells was measured by hybridization with <sup>32</sup>P-labeled specific oligonucleotides as described elsewhere (Ivanov *et al.*, 1992; Gigova *et al.*, 1989; Ivanov *et al.* 1993).

Secondary structure of RNA was studied using the computer program PC Gene (IntelliGenetics).

## Results and Discussion

Epsilon (ε) sequence is one of the most extensively studied bacterial enhancers. Comparing homology between *E. coli* and *A. tumefaciens* 16S rRNAs we have found a variable region at nucleotides 458–467 coinciding with the εBS (Fig. 2). This made it possible to study the significance of the complementarity of ε to the corresponding εBS in 16S rRNA on its enhancing activity in vivo.

In a previous study we have found correlation between the **enhancing activity** of ε and its complementarity to the εBS in both *E. coli* and *A. tumefaciens* cells (Golshani *et al.*, 2000a). It should be stressed, however, that **enhancement** does not necessarily mean **initiation of translation**. That is why the aim of this study is to check whether the ε as well as some other nucleotide sequences known to be effective enhancers or initiators of translation in *E. coli* can also independently initiate translation of CAT mRNA in *A. tumefaciens* cells.

To this end the following series of synthetic RBSs was prepared (Fig. 1A): SD<sub>cons</sub>, containing a SD consensus (AAGGAGGT) sequence; ε<sub>Ec16</sub>, bearing the ε natural (TTAACTTTA) sequence (Olins and Rangwala, 1989) placed 16 nucleotides before the initiation codon; ε<sub>A18</sub> and ε<sub>A116</sub>, an ε derivative sequences matching *A. tumefaciens* εBS and carrying a spacer of 8 and 16 nucleotides to the initiation codon respectively; ε<sub>II</sub>, an ε derivative sequence in which the complementarity to *A. tumefaciens* εBS is extended from 9 to 16 nucleotides; Ω, the 5' non-translated region of TMV RNA (Ivanov *et al.*, 1992; Ivanov *et al.*, 1995); PMV<sub>NTR</sub>, the 5' non-translated region of papaya mosaic virus (PMV) RNA (Sit and AbouHaidar, 1993); CYMV<sub>NTR</sub>, the 5' non-translated region of clover yellow mosaic virus (CYMV) RNA (Holy and AbouHaidar, 1993); ε<sub>Ec/SD</sub>, carrying a natural

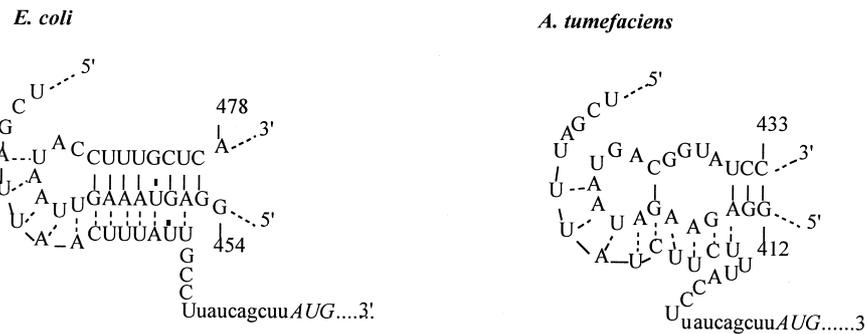


Fig. 2. Putative base pairing (dashed lines) between the helical domains #17 in *E. coli* and *A. tumefaciens* 16S rRNA (central parts) and the 5' ends of CAT mRNAs (outer parts) transcribed from the constructs  $\epsilon_{Ec16}$  (left) and  $\epsilon_{At16}$  (right).

$\epsilon$  sequence (Olins and Rangwala, 1989) combined with a SD consensus sequence;  $\epsilon_{At/SD}$ , an  $\epsilon$  derivative sequence matching *A. tumefaciens*  $\epsilon$ BS combined with a SD consensus sequence;  $\Delta\epsilon/SD$ , a neutral construct devoid of both  $\epsilon$  and SD sequences (negative control). The RBS sequences were first cloned in the expression vector pBRP<sub>1</sub>CAT (containing a strong constitutive promoter P<sub>1</sub> and a reporter *cat* gene devoid of a translation initiation sequence) (Golshani *et al.*, 1997) and then re-cloned into the binary vector pGA643 (Fig. 1B). The translation initiation activity of all RBSs was evaluated by the yield of CAT in both *E. coli* and *A. tumefaciens* cells. Taking into consideration that minor changes in gene structure could interfere with the efficiency of transcription, the yield of mRNA was also measured and the yield of protein was related to that of corresponding mRNA.

Table I shows that  $\epsilon$  in combination with the SD consensus sequence (as in the constructs  $\epsilon_{Ec/SD}$  and  $\epsilon_{At/SD}$ ) is an efficient enhancer of translation in *E. coli* as well as in *A. tumefaciens*. In the absence of a SD sequence ( $\epsilon_{Ec16}$  and  $\epsilon_{At16}$ ), however, it is capable of initiating translation in *E. coli* but not in *A. tumefaciens* cells. Our attempts to obtain translation initiation activity of  $\epsilon$  alone (in the absence of a SD) in *A. tumefaciens* cells by changing the distance to the initiation codon (as in  $\epsilon_{At8}$ ) or extending the complementarity to 16S rRNA from 9 to 16 nucleotides (as in  $\epsilon_{At11}$ ) have all failed. Similar results were obtained also with the rest of the viral enhancers ( $\Omega$ , PMV<sub>NTR</sub> and CYMV<sub>NTR</sub>) used in this study. They all were active in *E. coli* and inactive in *A. tumefaciens* cells (Table I).

Table I. Chloramphenicol acetyltransferase (CAT) yield in *E. coli* and *A. tumefaciens* obtained with different translation initiation sequences (RBS).

Translation Initiation sequence	<i>E. coli</i>		<i>A. tumefaciens</i>	
	U/mg <sup>a)</sup>	Relative yield <sup>b)</sup>	U/mg <sup>a)</sup>	Relative yield <sup>b)</sup>
SD <sub>cons</sub>	1009	1.00	2109	1.00
$\epsilon_{Ec16}$	716	0.71	63	0.03
$\epsilon_{At16}$	322	0.32	84	0.04
$\epsilon_{At8}$	100	0.10	84	0.04
$\epsilon_{At11}$	514	0.51	84	0.04
$\Omega$	232	0.23	63	0.03
PMV <sub>NTR</sub>	413	0.41	84	0.04
CYMV <sub>NTR</sub>	292	0.29	63	0.03
$\epsilon_{Ec/SD}$	1755	1.74	3943	1.87
$\epsilon_{At/SD}$	1190	1.18	5715	2.71
$\Delta\epsilon/SD$	110	0.11	84	0.04

<sup>a)</sup> The yield of CAT is related to that of mRNA determined as described in Materials and Methods. 1U = 1  $\mu$ mol/lmin acetylated chloramphenicol.

<sup>b)</sup> The yield of CAT is related to that obtained with the referent construct SD<sub>cons</sub>. For constructs see Fig. 1.

$\Omega$  sequence is believed to initiate translation in *E. coli* via base pairing with nucleotides 1344–1355 located at the helical domain #43 of *E. coli* 16S rRNA (Gallie and Kado, 1989; Ivanov *et al.*, 1995). This region is conserved in both *E. coli* and *A. tumefaciens* 16S rRNAs. The other two sequences (PMV<sub>NTR</sub> and CYMV<sub>NTR</sub>) show negligible (4–5 nucleotide long) complementarity with multiple places in 16S rRNA and the mechanism by which they initiate translation of CAT mRNA in *E. coli* cells remains obscure.

The fact that the four nucleotide sequences ( $\epsilon$ ,  $\Omega$ , PMV<sub>NTR</sub> and CYMV<sub>NTR</sub>) used in this study

are active as initiators of translation in *E. coli* and are inactive in *A. tumefaciens* cells indicates that the complementarity to 16S rRNA only is not a sufficient requirement for the initiation of mRNA translation in the latter microorganism. In order to understand the inability of *A. tumefaciens* to initiate translation of mRNAs devoid of SD sequences, we studied the role (if any) of the S1 ribosomal protein in this process. The S1 protein is presumed to account for the species specificity of translation in *E. coli* (Higo *et al.*, 1982) under both in vivo (Sorensen *et al.*, 1998) and in vitro (Suryanarayana and Subramanian, 1983; Subramanian, 1983) conditions. Cross-linking experiments have proven a functional interaction between S1 and the 5' untranslated region of certain mRNAs (Boni *et al.*, 1991). Tzareva *et al.* (1994) found a key role of S1 in the recognition of  $\Omega$  sequence when used as a translational initiator.

In *E. coli* the S1 protein is encoded by the *rpsA* gene. However, in *A. tumefaciens* such a gene has not been described so far. S1 protein genes in other bacteria belonging to *Rhizobiaceae* show 47% amino acid identity to that of *E. coli* and seem to be functionally interchangeable. It has been shown that *Rhizobium melilotii* S1 protein provides complementation to a temperature sensitive mutation in the *E. coli* S1 gene when expressed in *E. coli* cells (Schnier *et al.*, 1988).

The missing information about the S1 protein in *A. tumefaciens* led us to test total protein extracts from *A. tumefaciens* for immunoreactivity with antibodies raised against *E. coli* protein S1. The Western-blot analysis revealed a low intensity band of 55 kD (data not shown). The molecular mass of this putative S1 protein was found lower than that of *E. coli* (61 kD). Taking into consideration the importance of S1 for the accuracy of translation in *E. coli*, it could be speculated that the inability of *A. tumefaciens* ribosomes to translate mRNAs devoid of SD initiators might be

related to the size and structural differences of their S1 protein. To prove this, we have co-expressed the *rpsA* gene with the reporter *cat* gene placed under the translational control of any of the above four ( $\epsilon$ ,  $\Omega$ , PMV<sub>NTR</sub> or CYMV<sub>NTR</sub>) sequences in *A. tumefaciens*. Although the *rpsA* protein was found in the *A. tumefaciens* cells (judging by the western-blot analysis), we failed to detect CAT activity. The latter could be explained in two ways: (i) inability of *E. coli* S1 protein to substitute for (or to function together with) the *A. tumefaciens* S1 protein and (ii) neither complementarity to 16S rRNA nor the presence of S1 protein (from *E. coli*) is sufficient for the efficient initiation of translation of mRNAs devoid of SD sequences in *A. tumefaciens* cells.

### Conclusions

From the data presented in this report we can conclude that: (i) the alternative (non-SD) initiation of translation is a complex process involving other factors rather than simply mRNA-rRNA base pairing and mRNA-protein S1 interaction; (ii) the *A. tumefaciens* ribosomes are unable to translate mRNAs devoid of SD sequences; (iii) the  $\epsilon$  sequence can play the role of independent translational initiator in *E. coli* but not in *A. tumefaciens* cells; (iv) there are substantial differences in the mechanism of translation initiation and (possibly) in the ribosome structure of the two gram negative microorganisms.

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