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A 5′ stem–loop and ribosome binding but not translation are important for the stability of *Bacillus subtilis aprE* leader mRNA

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The *Bacillus subtilis aprE* leader is a determinant of extreme mRNA stability. The authors examined what properties of the *aprE* leader confer stability on an mRNA. The secondary structure of the *aprE* leader mRNA was analysed *in vitro* and *in vivo*, and mutations were introduced into different domains of an *aprE* leader–*lacZ* fusion. The half-lives of the corresponding transcripts were determined and β-galactosidase activities were measured. Removal of a stem–loop structure at the 5′ end or diminishing the strength of the RBS reduced the half-lives from more than 25 min to about 5 min. Interfering with translation by abolishing the start codon or creating an early stop codon had no or little effect on mRNA stability. The authors conclude that a 5′ stem–loop and binding of ribosomes are necessary for the stability of *aprE* leader mRNA. The present results, together with a number of other data, suggest that translation of a *B. subtilis* mRNA is generally not important for its stability; the situation seems different in *Escherichia coli*. It is further concluded that the calculated strength of a *B. subtilis* RBS cannot be used to predict the stability of the corresponding transcript.

Keywords: mRNA degradation, stability determinants

INTRODUCTION

The steady-state amount of an mRNA in a cell is a function of its rate of synthesis and its rate of degradation. Studies of mRNA degradation in *Escherichia coli* and other bacteria have shown that this is a highly regulated process, giving half-lives of individual mRNAs from less than one min to more than 30 min (Belasco, 1993). The half-lives of some mRNAs are influenced by growth stage and growth conditions (Paesold & Krause, 1999; Vytvytska *et al.*, 1998).

The general mechanism of mRNA degradation in *E. coli* is quite well understood (Grunberg-Manago, 1999; Rauhut & Klug, 1999). Degradation proceeds in a 5′ to 3′ direction by successive endonucleolytic cleavages performed by RNase E (Cohen & McDowall, 1997) or less commonly RNase III (Court, 1993; Régnier & Grunberg-Manago, 1990). The resultant mRNA fragments are further processed by the 3′ to 5′ exoribonucleases RNase II and PNPase (Spickler & Mackie, 2000) and finally degraded to mononucleotides by an oligoribonuclease (Ghosh & Deutscher, 1999).

Much less is known about mRNA degradation in other bacteria. Nevertheless, studies in *Bacillus subtilis* have revealed significant differences in the degradation mechanism between this bacterium and *E. coli*. The two bacteria have different arsenals of ribonucleases, one important distinction being that there is no RNase E homologue in *B. subtilis*. While the 5′ region appears to be the most important determinant of mRNA stability in both bacteria, the same mRNA species can show different degradation patterns in *B. subtilis* and *E. coli* (Persson *et al.*, 2000).

The *B. subtilis aprE* gene encodes the alkaline protease subtilisin (Ferrari *et al.*, 1988). Transcription of *aprE* is under AbrB/Spo0A control (Strauch & Hoch, 1993) and the gene is only expressed in stationary-phase bacteria. The *aprE* mRNA is unusually stable with a half-life exceeding 25 min. We have recently shown that the determinants for *aprE* mRNA stability are located in the 5′ untranslated 58 nt long leader sequence. *aprE* leader–*lacZ* fusion mRNA has a half-life of $\geqslant$25 min also in exponentially growing bacteria, showing that the ex-

Abbreviation: DMS, dimethyl sulphate.

treme stability conferred by the *aprE* leader is not growth phase dependent (Hambraeus *et al*., 2000).

In the present work we have examined what properties of the *aprE* leader confer stability on an mRNA molecule. Our results show that a stem–loop structure at the 5′ end together with an intact RBS are important for the stability of the mRNA. However, whether the mRNA is translated or not has little or no effect on its half-life.

METHODS

Bacteria and plasmids. These are listed in Table 1.

Growth of bacteria. Bacteria were kept on TBAB plates and liquid cultures were grown in LB at 37 °C on a rotary shaker at 200 r.p.m. For measurement of β-galactosidase activities and extraction of RNA, bacteria were grown to an OD_{600} of about 1·0 (mid-exponential phase). For determination of mRNA half-lives, the bacteria were incubated with 100 mg rifampicin l^{-1} for various times before samples were taken.

Genetic techniques. *B. subtilis* was grown to competence as described by Arwert & Venema (1973). *E. coli* was made competent as described by Mandel & Higa (1970).

Construction of strains. Plasmid pLUS2 is a derivative of pMD432 into which has been inserted a *glpD* promoter–*aprE* leader–*lacZ* translational fusion. Transformation of *B. subtilis* BR95 with pLUS2 gave rise to strain LUS2 where the fusion has been integrated into the *amyE* locus (Fig. 1). Further, pLUS2 was used as template for PCRs with modified primers to introduce directed mutations into the *aprE* leader (Hambraeus *et al*., 2000). The same protocol was now employed to construct additional plasmids and strains with mutated *aprE* leader–*lacZ* fusions. The plasmid constructs were verified by DNA sequencing. The mutations introduced are shown in Fig. 2, the primers used are listed in Table 2 and the resultant strains in Table 1.

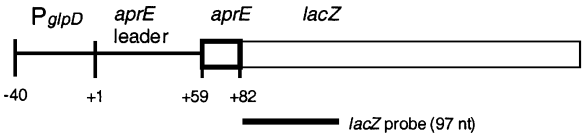

Fig. 1. Schematic representation of the insert of *B. subtilis* LUS2. +1 indicates the transcription start point.

β-Galactosidase activity. β-Galactosidase activity was detected on TBAB plates containing 40 mg X-Gal l^{-1}. β-Galactosidase in liquid cultures was assayed according to Miller (1972) as described by Glatz *et al*. (1998).

RNA techniques. Total RNA was extracted as described by Putzer *et al*. (1992) with some modifications. A 15 ml culture sample was added to a centrifuge tube filled to one-third with ice. The sample was centrifuged (5000 r.p.m. for 10 min) and the pellet was resuspended in 0·4 ml ice-cold TES buffer (50 mM Tris/HCl, pH 7·5; 5 mM EDTA; 50 mM NaCl) and transferred to a tube containing 0·6 ml acid phenol, 0·15 ml chloroform and 0·8 ml 0·1 mm silica beads. The mixture was vortexed in a Mini Bead Beater (Biospec Products) at full speed for 80 s and then centrifuged at 5000 r.p.m. for 5 min. The aqueous phase was recovered and extracted with 0·6 ml acidic phenol and 0·15 ml chloroform and then once more with 0·7 ml chloroform. Total RNA was finally precipitated from the aqueous phase with 1/10 vol. 3 M NaAc, pH 4·8, and 2·5 vols 95% ice-cold ethanol. After centrifugation and washing with ice-cold 70% ethanol, the pellet was resuspended in 0·2 ml diethyl-pyrocarbonate-treated water. The quality of the RNA was controlled by electrophoresis in a 0·8% agarose gel with ethidium bromide.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/phenotype	Source/reference
***E. coli* strains**		
XL-1 Blue	*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac*F′[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^R)]	Bullock *et al*. (1987)
***B. subtilis* strains**		
BR95	*ilvC1 pheA1 trpC2*	Dept. Microbiology, Lund University
LUS2	BR95 with insertion of a *glpD* promoter–*aprE* leader–*lacZ* fusion into *amyE*; Cm^R	Hambraeus *et al*. (2000)
LUS7*	As LUS2 but with A and T substitutions at +23 and +27 in the *aprE* leader	This work
LUS8*	As LUS2 but with an A substitution at +46 in the *aprE* leader	This work
LUS9*	As LUS2 but with A substitutions at +46 and +48 in the *aprE* leader	This work
LUS11*	As LUS2 but with the start codon GUG replaced by AAA	This work
LUS12*	As LUS2 but with the fifth codon replaced by UAA	This work
Plasmids		
PMD432	Cm^R Ap^R; Δ*amyE*::′*lacZ*	Dahl & Meinhof (1994)
pLUS2	Derivative of pMD432; Δ*amyE*::*glpD* promoter–*aprE* leader–*lacZ* fusion	Hambraeus *et al*. (2000)

* See Fig. 2.

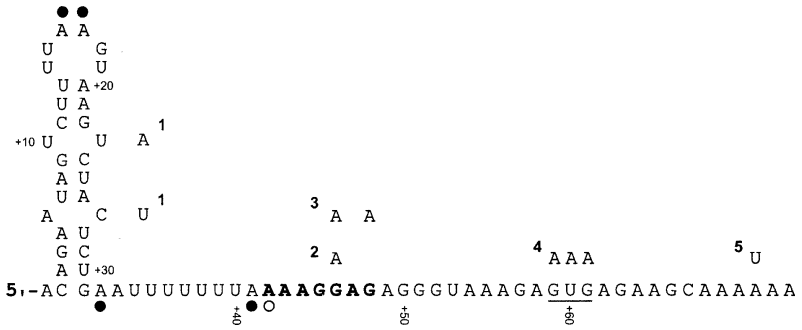

Fig. 2. Proposed structure of the *aprE* leader mRNA. The RBS is in bold face. The start codon is underlined. Mutations introduced are: 1, U at +23 changed to A and C at +27 changed to U (LUS7); 2, G at +46 changed to A (LUS8); 3, Gs at +46 and +48 changed to As (LUS9); 4, the start codon GUG replaced by AAA (LUS11); 5, the fifth codon, AAA, replaced by the stop codon UAA (LUS12). ● and ○ indicate strong and weak methylation, respectively, in the experiment shown in Fig. 3.

Table 2. Primer sequences

Name	Sequence (5′–3′)*
aprEconst	TTG GAT CCT TTT AAA TAA AGT AAT ACT ATG GTA TAA TGG TTA CAC AGA ATA GTC TTT TAA GTA AGT CTA CTC TG
aprEconstS	TTG GAT CCT TTT AAA TAA AGT AAT ACT
aprEBam2	CAA GGA TCC GAT CCA CAA TTT TTT GCT TCTC
aprEBulge	GTC TTT TAA GTA AGA CTA TTC TGA ATT TTT TTA
aprEBulgeinv	TAA AAA AAT TCA GAA TAG TCT TAC TTA AAA GAC
aprERBS1	GAA TTT TTT TAA AAG AAG AGG GTA AAG AGT GAG
aprERBS1inv	CTC ACT CTT TAC CCT CTT CTT TTA AAA AAA TTC
aprERBS2	GAA TTT TTT TAA AAG AAA AGG GTA AAG AGT GAG
aprERBS2inv	CTC ACT CTT TAC CCT TTT CTT TTA AAA AAA TTC
aprEnostart	GGC GGA TCC GAT CCA CAA TTT TTT GCT TCT TTT TCT TTA CCC TC
aprEstop	GGC GGA TCC GAT CCA CAA TTA TTT GCT TCT CAC TCT TTA CCC
lacZseq	GTT TTC CCA GTC ACG ACG TTG

* The mutations introduced by the primers are underlined.

Electrophoresis of RNA for Northern blots was done as described by Thomas (1980). RNA (10 μg) was added to each well. The RNA was blotted onto Hybond-N filters (Amersham). A single-stranded radioactive DNA probe for Northern blots (Fig. 1) was generated as previously described (Hambraeus *et al*., 2000). After hybridization, the radioactivity of the bands was quantified using a PhosphorImager (Molecular Dynamics). Primer extension analysis was performed according to the method of Ayer & Dynan (1988). The primer used was lacZseq which is complementary to the 5′ end of the *lacZ* part of the *aprE* leader–*lacZ* fusion mRNA.

Treatment of cells with dimethyl sulphate (DMS) was performed as described by Mayford & Weisblum (1989). Culture samples of 15 ml were transferred to a tube and 0·4 ml DMS was added. After 4 min vigorous shaking, 10 ml ice-cold TME buffer (100 mM Tris/HCl, pH 7·5; 100 mM β-mercaptoethanol; 5 mM EDTA) was added and RNA was extracted as described above. In parallel, cells that had not been incubated with DMS were treated in the same way and used as control.

RESULTS

The secondary structure of the *aprE* leader mRNA

The secondary structure of an RNA molecule can be estimated by using algorithms which search for the energetically most favoured structure. However, this procedure often yields several energetically similar but structurally different foldings of the same molecule. Moreover, since an RNA molecule may start to fold before its synthesis is completed, it could be locked *in vivo* in an energetically less favoured structure, which can be very different from the one(s) suggested by *in silico* experiments.

To facilitate studies on the *aprE* leader mRNA, Hambraeus *et al*. (2000) fused the *aprE* leader sequence to *lacZ* and replaced the *aprE* promoter, which is active only in stationary phase cells, with the constitutive *B. subtilis glpD* promoter. The construct was integrated into the *amyE* locus of the *B. subtilis* chromosome (strain LUS2, Fig. 1). The *aprE* leader–*lacZ* fusion mRNA was as stable as the native *aprE* mRNA, showing that the *aprE* leader contains the stability determinants.

To get information about the *in vivo* secondary structure of the *aprE* leader mRNA, an exponentially growing culture of LUS2 was treated with DMS, which preferentially methylates unpaired adenine and cytosine residues. RNA was then extracted and used in primer extension experiments with a primer specific for the *aprE* leader–*lacZ* mRNA. Methylated nucleotides act as stop signals for reverse transcriptase and thus the reaction is prematurely terminated at the positions of

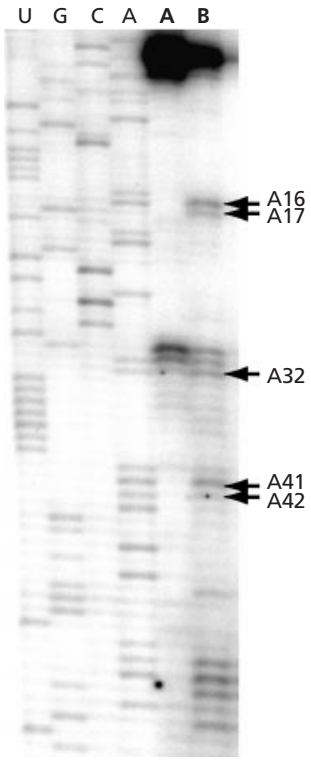

Fig. 3. Primer extension analysis of the *aprE* leader–*lacZ* mRNA methylated *in vivo* by DMS. Total RNA was extracted from growing LUS2 treated with DMS (**B**) or untreated (**A**). Methylated adenines are indicated.

modified nucleotides (Fig. 3). A probable structure of the *aprE* leader mRNA predicted by the mfold programme (version 3.1, Mathews *et al*., 1999; Zuker *et al*., 1999) has a stable stem–loop structure at the very 5′ end (Fig. 2). Two of the six unpaired nucleotides in the loop are adenines. These residues, but no others, show up as stop signals in the DMS-treated RNA, supporting the presence of a stem–loop at the 5′ end. The stem–loop contains two bulges, one of which is formed by an adenine and a cytosine. These nucleotides were not seen to be methylated which we think may be because the small bulge is not readily accessible to DMS. A short stem–loop in the RBS region is also predicted and the primer extension experiments suggest methylation of three adenine residues at positions 41–43 which are believed to be part of the RBS. However, the loading of ribosomes on the mRNA must interfere with secondary structure formation in the RBS region and it could be assumed to be mostly single-stranded or melted *in vivo* upon engagement with the 16S rRNA 3′ end (de Smit & van Duin, 1990). Fig. 2 shows the structure of the *aprE* leader mRNA which is best compatible with the combined results of the computer predictions and the DMS experiments.

Effects of directed mutations on the stability of *aprE* leader–*lacZ* mRNA

One can distinguish three structural/functional domains in the *aprE* leader–*lacZ* mRNA: the 5′ stem–loop, the RBS and the start of the coding region. Mutations were

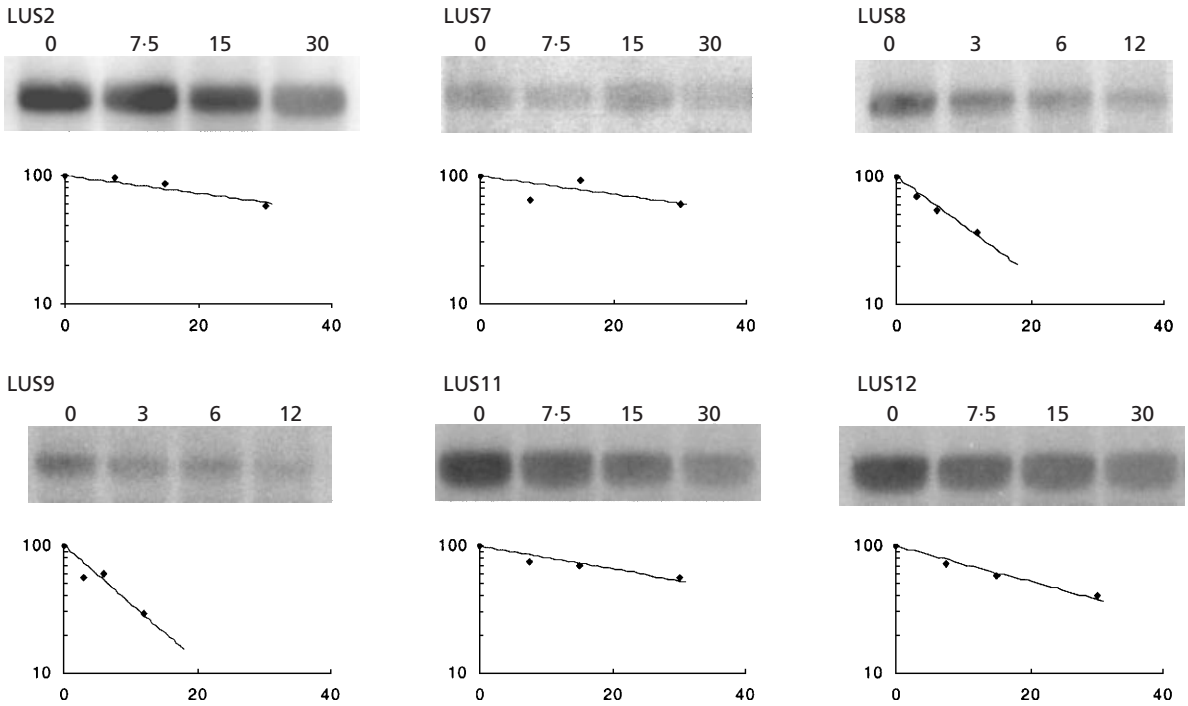

Fig. 4. Northern blot analysis and calculation of half-lives of the *aprE* leader–*lacZ* mRNAs. The bacteria were grown to OD_{600} 1·0, rifampicin was added and RNA was extracted at the times indicated (min). The *y*-axes of the graphs indicate % remaining mRNA. The experiments were repeated at least twice with similar results.

Table 3. Half-lives and steady-state levels of *aprE* leader–*lacZ* mRNAs and pertaining β-galactosidase activities

Strain	Mutation	mRNA half-life (min)	Effective half-life (min)*	Relative effective half-life	Relative steady-state level†	Relative β-galactosidase activity‡
LUS2	None	⩾25	⩾14	1	1	1
LUS7	Removal of two bulges in the 5′ end stem–loop	⩾25	⩾14	1	0·07	0·2
LUS8	G of RBS (+46) changed to A	6	5	0·4	0·4	0·2
LUS9	Two Gs of RBS (+46 and +48) changed to As	6	5	0·4	0·3	0·01
LUS11	Start codon GUG changed to AAA	18	11	0·8	0·9	<0·01
LUS12	Fifth codon changed to the stop codon UAA	25	14	1	0·9	<0·01

* The effective half-life ($T_{1/2E}$) is derived from the measured half-life $T_{1/2M}$ and the dilution due to cell growth (Belasco & Brawerman, 1993): $T_{1/2E} = [(T_{1/2M})^{-1} + (T_D)^{-1}]^{-1}$. The generation time ($T_D$) was 30 min.

† Determined from Northern blots (data not shown).

‡ The β-galactosidase activity of LUS2 corresponded to 2 μmol ONPG hydrolysed min^{-1} (mg protein)$^{-1}$. The extinction coefficient used was $4{\cdot}5 \times 10^3$ M^{-1} cm^{-1}. The values represent the mean of at least two independent experiments. The deviation from the mean was less than 20%.

introduced into each of these domains (Fig. 2) and the constructs were integrated into the *amyE* locus of the *B. subtilis* chromosome. The effects of the mutations on the stability and translation of the various mRNAs were then determined.

We have previously shown that two nucleotide substitutions, which are predicted to disrupt the stem–loop at the 5′ end, lead to at least a fivefold reduction of the half-life of the *aprE* leader–*lacZ* transcript. A similar reduction in half-life is also seen following removal of the stem–loop by deletion of nt +1 to +25 (Hambraeus *et al.*, 2000). To test the possibility that the bulges of the stem are a binding site for a protein that protects the mRNA from cleavage, we exchanged the nucleotides at positions +23 and +27 such that the bulges disappeared (Fig. 2). In the resultant strain, LUS7, the half-life of the *aprE* leader–*lacZ* transcript was found to be the same as that of the wild-type, i.e. 25 min or longer (Fig. 4 and Table 3).

To interfere with ribosome binding, we mutated the RBS by changing the G at position +46 to an A to give strain LUS8 and the Gs at +46 and +48 to As to give strain LUS9 (Fig. 2). The calculated free energies of interaction (see legend of Table 4) between the RBS and the 3′ end of 16S rRNA of the wild-type and mutants are (kcal mol^{-1}): LUS2, −8·9; LUS8, −7·3; and LUS9, −5·2. The half-lives of both mutant *aprE* leader–*lacZ* mRNAs were about 6 min compared to 25 min for the wild-type mRNA (Fig. 4 and Table 3).

Finally, mutations were introduced into the coding region of the *aprE* leader–*lacZ* mRNA. In strain LUS11, the GUG start codon was changed to AAA. In strain LUS12, a U was introduced in the first position of the fifth codon, changing it to the stop codon UAA. These mutations had little or no effect on the half-lives of the respective *aprE* leader–*lacZ* transcripts, which were about 18 min for LUS11 and about 25 min for LUS12 (Fig. 4 and Table 3).

The steady-state level of an mRNA is a function of the rate of synthesis and the rate of removal. The rate of removal is often, for the sake of simplicity, taken to be the same as the rate of degradation of the mRNA, i.e. it is calculated from the half-life of the mRNA. However if the half-life of an mRNA is close to, or longer than, the generation time, the 'effective' half-life of the mRNA is derived from the half-life and the dilution due to cell growth (see legend of Table 3). The different *aprE* leader–*lacZ* fusions are all preceded by the constitutive *glpD* promoter. They should therefore be transcribed with the same efficiency and the steady-state levels of the respective transcripts should correspond to their effective half-lives, i.e. a decreased effective half-life should lead to a lowered steady-state level. The steady-state levels of the wild-type and mutant *aprE* leader–*lacZ* transcripts were measured and the values showed good correspondence between relative steady-state level and relative effective half-life for all strains except LUS7 (Table 3). In this strain the steady-state level was less than 10% of the wild-type level although the half-lives of the two transcripts were the same. By removing the bulges in the wild-type leader mRNA, we have created a strong transcriptional stop signal consisting of a 'perfect' stem–loop followed by a run of seven U residues. We suggest that the majority of the *aprE* leader–*lacZ* transcripts initiated in LUS7 are prematurely terminated at this stop signal.

Translation of wild-type and mutant *aprE* leader–*lacZ* transcripts

The previous experiments have shown that mutations affecting the RBS destabilize the *aprE* leader–*lacZ* transcript whereas mutations that affect translation have little effect on mRNA half-lives. To confirm that the effects of the mutations on translation were the expected ones, we measured β-galactosidase activity in extracts from exponentially growing cells. The results of these experiments are shown in Table 3. It can be noted that

Table 4. *B. subtilis* mRNAs with known half-lives

mRNA	Sequence*	ΔG_0 (kcal mol^{-1})	Half-life (min)	Reference
amyE	AAAUCAAAUAAGGAGUGUCAAGA AUG	−8·5	5	Hambraeus *et al.* (2000)
aprE	UUUUUAAAAGGAGAGGGUAAAGA GUG	−8·9	⩾25	Hambraeus *et al.* (2000); Resnekov *et al.* (1990)
clpC	AAAAGACAGGAGGAUGAAUCGAU AUG	−10·1	2	Allmansberger (1996)
cspB	UGAUCGCUUUAGGAGGAAAUUUC AUG	−11·2	2	Kaan *et al.* (1999); Graumann *et al.* (1997)
cspC	UUACACACUAGGAGGCAACAAAA AUG	−10·0	2	Kaan *et al.* (1999); Graumann *et al.* (1997)
ctc	AAAACGACAAGAGGAUGGUGAAU AUG	−9·4	<5	Jürgen *et al.* (1998)
glpD	CGUGACAACAAGGAGGAAACGUA AUG	−12·2	2·3†	Glatz *et al.* (1996)
groES	AUACUAUUGAGGAGGUUAUUUCA UUG	−12·7	5	Yuan & Wong (1995)
gsiB	CACCAUUAAAGGAGGAAUUCAAA AUG	−12·2	20	Jürgen *et al.* (1998)
gspA	AACAAAAAGGGGAGAUGAAUACCA UUG	−8·5	5–7	Jürgen *et al.* (1998)
odhAB	UUUAAGUUGGGGGUAAUAUUCAA AUG	−9·7	1·5	Resnekov *et al.* (1992)
sacR	ACAUAAAAAAGGAGACAUGAACG AUG	−8·9	0·6–2·5	Pereira *et al.* (2001)
sdh	CUUAUCAAACAGGGGGUAAAGUA AUG	−11·2	2·6	Melin *et al.* (1989)
thrS	UGCAAAAAAAAGGAGUGACAAAG AUG	−8·3	2	Condon *et al.* (1997)
xynA	AAAAAUAUUAUAGGAGGUAACAU AUG	−12·2	8	Allmansberger (1996)

* The 23 nt upstream of the start codon were aligned with the 3′ end of the 16S rRNA sequence (UUUCCUCCA) and the free energy was calculated using the programme RNA structure 3.6. The nucleotides that are predicted to be involved in the hybridization with 16S rRNA are underlined.

† The half-life was determined at 45 °C. At 32 °C the half-life was 4·5 min.

LUS8 with one mutation in the RBS had about 20% of the wild-type β-galactosidase activity and about 40% of the wild-type steady-state amount of *aprE* leader–*lacZ* mRNA. Thus, the translational efficiency of *aprE* leader–*lacZ* transcripts in LUS8 was about half of that of the wild-type (LUS2). LUS9 with two mutations in the RBS showed a very low β-galactosidase activity but the half-life of its *aprE* leader–*lacZ* mRNA was the same as that of the LUS8 mRNA. No β-galactosidase activity was found in LUS11 where formation of the translational initiation complex must be severely impaired or in LUS12 carrying a premature stop codon. Still these mRNAs have a half-life close to that of the wild-type mRNA. The enzyme activities correlated well with the amount of β-galactosidase protein produced in the different strains as measured in Western blots (data not shown).

DISCUSSION

The *B. subtilis aprE* leader sequence contains determinants for extreme mRNA stability. The aim of this work was to define those determinants.

The first step in analysing the *aprE* leader mRNA was to determine its secondary structure. From computer predictions and *in vivo* DMS methylation experiments we derived the structure shown in Fig. 2, which has a stem–loop at its 5′ end.

Specific changes were introduced into different domains of the *aprE* leader–*lacZ* fusion mRNA, the first target being the 5′ stem–loop. Deletion of nt +1 to +25 has been shown to result in a fivefold reduction of the half-life of *aprE* leader mRNA (Hambraeus *et al.*, 2000), which indicates that the stem–loop is an important stability determinant. The two bulges of the stem–loop are apparently not required for stability (e.g. by binding a protecting protein) since their removal did not affect the half-life of the *aprE* leader–*lacZ* transcript.

A stem–loop at the very 5′ end of the *E. coli ompA* mRNA is important for stability. Addition of a short single-stranded region to the 5′ end destabilizes the transcript (Arnold *et al.*, 1998; Emory *et al.*, 1992). Most likely this is because a single-stranded end facilitates or is required for binding of RNase E and, consequently, introduction of the first (rate-determining) endonucleolytic cleavage (Mackie, 2000). No RNase E homologue is known in *B. subtilis* (Kunst *et al.*, 1997), although the existence of a similar enzyme has been postulated (Condon *et al.*, 1997). It is possible that also the *aprE* leader mRNA stem–loop interferes with binding of an endonuclease, thus delaying an initial step in degradation of the mRNA.

Changing a G to an A in the RBS (LUS8) led to a fivefold decreased half-life of the *aprE* leader–*lacZ* mRNA. The amount of β-galactosidase produced by this mutant was also reduced about fivefold. Considering that there is less *aprE* leader–*lacZ* mRNA to be translated in LUS8 (40% of the wild-type steady-state amount), we can

estimate that the mutation of LUS8 reduces the translational efficiency to about 50%. Changing two Gs to As in the RBS (LUS9) caused no further decrease in the stability of the mRNA but the translational efficiency was reduced to a few per cent. Thus, reducing the translational efficiency about 30-fold by mutating the RBS had no effect on mRNA stability.

An untranslated mRNA leader sequence from the *Bacillus thuringiensis cryIIIA* gene has been shown to stabilize in *B. subtilis* a *lacZ* gene fused to the 3′ end of the leader sequence. Stabilization requires the 129 nt at the 3′ end of the leader mRNA. A strong RBS begins at −125 and is separated by one nucleotide from an AUG which, however, does not seem to be part of an ORF. The stabilizing effect is suggested to depend on binding of a 30S ribosomal subunit and to be independent of translation of the downstream *lacZ* gene (Agaisse & Lereclus, 1996). A contribution of secondary structures in the 129 nt sequence was not considered. Similarly, a polypurine sequence from *B. subtilis* phage SP82 has been reported to stabilize an *ermC* or a *lacZ* gene fused to its 3′ end. Stabilization depended on an RBS which precedes a phage ORF but translation of the fused genes was not required (Hue *et al*., 1995). Earlier work on the *ermA* gene in *B. subtilis* showed that the stalling of ribosomes at a short ORF in the leader mRNA stabilized the *ermA* mRNA. The stalled ribosomes were suggested to block progression of mRNA degradation in a 5′ to 3′ direction (Sandler & Weisblum, 1989). Abolishing the start codon or introducing an early stop codon in the *aprE* leader–*lacZ* fusion had little or no effect on mRNA stability. Neither of these mutants produced detectable amounts of β-galactosidase activity or protein. We conclude that translation has no effect on the stability of the *aprE* leader–*lacZ* mRNA.

In contrast, the stability of an *E. coli* mRNA generally seems to depend not only on ribosome binding but also on translation. Introduction of an early stop codon in the *ompA* gene or the *bla* gene destabilizes the respective transcripts indicating that translation is important for their stability (Nilsson *et al*., 1987). Increasing the speed of transcription of a gene (Joyce & Dreyfus, 1998) or stalling ribosomes at artificially introduced rare codons (Deana *et al*., 1998) has also been shown to destabilize a transcript in *E. coli*. In neither case was initial binding of ribosomes to the RBS impaired but less of the mRNA was covered by ribosomes. A recent study of the regulation of the *E. coli thrS* gene also suggests a strong correlation between translation and stability of the *thrS* transcript (Nogueira *et al*., 2001). However, it has been claimed that also in *E. coli*, an efficient RBS, irrespective of translation of downstream sequences, can be sufficient to protect a transcript from rapid degradation (e.g. Wagner *et al*., 1994).

Although no general rule can be formulated concerning the importance of translation for mRNA stability in eubacteria, available facts all suggest that binding of ribosomes is important both in *B. subtilis* and *E. coli*. Jürgen *et al*. (1998) determined the half-life of *B. subtilis gsiB* mRNA to be 20 min and weakening of the RBS decreased the half-life about fourfold. A comparison with two other sigma B-dependent mRNAs, *gspA* and *ctc*, also showed a correlation between the strength of the RBS and the stability of the mRNA. From these observations the general conclusion was drawn that the stronger the RBS, the more stable the corresponding mRNA.

We have surveyed the literature for determinations of *B. subtilis* mRNA half-lives. For each transcript we have calculated the energy of interaction between the RBS and the 3′ end of 16S rRNA based on the 23 nt preceding the start codon (Table 4). The stability of some of the mRNAs has been shown to be affected by, for example, stress (*xynA*; Allmansberger, 1996), a down-shift in temperature (*cspB* and *cspC*; Kaan *et al*., 1999) or growth phase (*sdh*; Melin *et al*., 1989) but, as far as we can deduce from the cited papers, the mRNA half-lives have been determined in bacteria growing at 37 °C.

Inspection of the data in Table 4 immediately reveals that there is no simple relation between the strength of interaction of the different RBSs with 16S rRNA and mRNA stability. In the present work we have shown that the native RBS is required but not sufficient for the high stability of the *aprE* leader–*lacZ* mRNA. Moreover, mutations that strongly reduced the strength of the RBS only reduced the stability of the mRNA to 6 min, which is longer than the mean mRNA half-life in *B. subtilis*. For the *groE* gene and segments of the *dnaK* operon in *B. subtilis*, the presence of an inverted repeat, CIRCE, and its positioning relative the RBS have been found to affect the stability of the mRNAs (Yuan & Wong, 1995; Homuth *et al*., 1999). An interaction between the RNA-binding antiterminator protein GlpP and the *glpD* leader mRNA in *B. subtilis* stabilizes *glpD* mRNA (Glatz *et al*., 1996). Clearly, the context of the leader sequence, possible secondary structures, or specific binding of proteins can be as important as the RBS in determining mRNA stability in *B. subtilis*.

We can conclude that the extreme stability of the *aprE* leader–*lacZ* mRNA is a function of a stem–loop structure at the 5′ end and a native RBS. The strength of interaction between the *aprE* RBS and 16S rRNA is less than average for the *B. subtilis* mRNAs presented in Table 4, emphasizing that the strength of an RBS cannot be used to predict the stability of a *B. subtilis* mRNA. When comparing *B. subtilis* with *E. coli*, it seems that translation is important for the stability of most *E. coli* mRNAs but unimportant for the stability of *B. subtilis* mRNAs. Whether this difference has any relation to the fact that an identical mRNA can have a very different half-life in the two species (Persson *et al*., 2000) is unknown.

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