The *aprE* leader is a determinant of extreme mRNA stability in *Bacillus subtilis*

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The *Bacillus subtilis aprE* gene encodes subtilisin, an extracellular proteolytic enzyme produced in stationary phase. The authors examined the stability of *aprE* mRNA and *aprE* leader–*lacZ* fusion mRNA. Both mRNAs were found to be unusually stable, with half-lives longer than 25 min, demonstrating that the *aprE* leader contains a determinant for extreme mRNA stability. The half-lives were the same in growing and stationary-phase cells. This contrasts with the findings of O. Resnekov *et al.* (1990) [*Proc Natl Acad Sci USA* 87, 8355–8359], which suggested a growth-phase-dependent mechanism for decay of *aprE* mRNA. The discrepancy is explained by the techniques used. Substitution of two bases or deletion of 25 nucleotides in the *aprE* leader led to a major difference in its predicted secondary structure and resulted in a fivefold reduction of the half-life of *aprE* mRNA. The authors also determined the half-life of *amyE* mRNA, which encodes α -amylase, another stationary-phase, excreted enzyme and found it to be around 5 min. This shows that extreme stability is not a general property of stationary-phase mRNAs encoding excreted enzymes.

Keywords: mRNA secondary structure, subtilisin, *amyE*, stationary phase

INTRODUCTION

The steady-state amount of mRNA in a cell is a function of its rate of synthesis and its rate of decay. The half-life of bulk mRNA in growing cells of *Escherichia coli* is about 3–5 min, but that of specific mRNA species can vary between less than 1 min and more than 30 min (Belasco, 1993). Furthermore, the half-lives of some mRNAs vary depending on growth stage and growth conditions (Paesold & Krause, 1999; Vytvytska *et al.*, 1998). Thus, there exist specific mechanisms for the control of decay of various mRNA species. These mechanisms involve specific RNA–protein interactions and alternative mRNA secondary structures (Liu & Romeo, 1997; Vytvytska *et al.*, 1998; Yamanaka *et al.*, 1999).

Most of our ideas about bacterial mRNA degradation are based on experiments performed with *E. coli*. The 5′ untranslated leader of an mRNA often contains the major stability determinant(s) (Bechhofer, 1993). The rate-limiting step in mRNA degradation is thought to be an endonucleolytic cut in the 5′ part of the molecule, which ‘opens’ it for further endonucleolytic attacks towards the 3′ end. The resulting fragments are rapidly

degraded by 3′ to 5′ exoribonucleases (Spickler & Mackie, 2000). RNase E is the major endoribonuclease executing the rate-limiting attack (Cohen & McDowall, 1997). The general model for mRNA degradation probably applies to other bacteria but with important modifications in details. For instance, no RNase E homologue has been found in *Bacillus subtilis* (Kunst *et al.*, 1997) and different patterns of degradation have been found for the same mRNA species in *B. subtilis* and *E. coli* (Persson *et al.*, 2000).

When *B. subtilis* enters stationary phase, several new genetic programmes are switched on for sporulation, competence development (Lazazzera *et al.*, 1999) or production of extracellular enzymes (Ferrari *et al.*, 1993). The *aprE* gene encodes subtilisin, an extracellular proteolytic enzyme produced by stationary-phase cells (Ferrari *et al.*, 1988). Resnekov *et al.* (1990) measured the decay of *aprE* mRNA and found it to be extremely stable in stationary-phase cells, with a half-life of at least 25 min. This is not a general property of stationary-phase mRNA (Melin *et al.*, 1989; Resnekov *et al.*, 1992). Interestingly, when the bacteria were diluted into fresh medium and allowed to resume growth, the stability of *aprE* mRNA seemed to decrease four- to fivefold. These

Table 1. Bacterial strains and plasmids

Genotype/phenotype		Source/reference
<i>E. coli</i>		
XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA96 thi-1 relA1 lac F'[proAB⁺ lacI^a lacZΔM15 Tn10(Tet^R)]</i>	Bullock <i>et al.</i> (1987)
<i>B. subtilis</i>		
BR95	<i>ilvC1 pheA1 trpC2</i>	Dept of Microbiology, Lund University
LUS1	BR95 with insertion of an <i>aprE</i> promoter– <i>aprE</i> leader– <i>lacZ</i> fusion into <i>amyE</i> ; Cm ^R	This work
LUS2	BR95 with insertion of a <i>glpD</i> promoter– <i>aprE</i> leader– <i>lacZ</i> fusion into <i>amyE</i> ; Cm ^R	This work
LUS3	As LUS2 but with a deletion of the first 25 nt of the <i>aprE</i> leader	This work
LUS4	As LUS2 but with T substitutions at positions +31 and +32 in the <i>aprE</i> leader	This work
LUS5	BR95 with insertion of an <i>amyE</i> promoter– <i>amyE</i> leader– <i>lacZ</i> fusion into <i>thrC</i> ; MLS ^R	This work
LUS6	BR95 with insertion of a <i>glpD</i> promoter– <i>amyE</i> leader– <i>lacZ</i> fusion into <i>thrC</i> ; MLS ^R	This work
Plasmids		
pMD432	Cm ^R Ap ^R ; Δ <i>amyE</i> ::' <i>lacZ</i>	Dahl & Meinhof (1994)
pMD433	Km ^R Ap ^R ; Δ <i>amyE</i> ::' <i>lacZ</i>	Dahl & Meinhof (1994)
pDG1664	MLS ^R Ap ^R ; Δ <i>thrC</i>	Guerout-Fleury <i>et al.</i> (1996)
pLUM1041	Km ^R Ap ^R ; ' <i>lacZ</i> (<i>glpD</i> leader from BR95 cloned in front of and in-frame with <i>lacZ</i> in pMD433)	Glatz <i>et al.</i> (1998)
pLUS1	Derivative of pMD432; Δ <i>amyE</i> :: <i>aprE</i> promoter– <i>aprE</i> leader– <i>lacZ</i> fusion	This work
pLUS2	Derivative of pMD432; Δ <i>amyE</i> :: <i>glpD</i> promoter– <i>aprE</i> leader– <i>lacZ</i> fusion	This work
pLUS3	Derivative of pLUS2 with the first 25 nt of the <i>aprE</i> leader deleted	This work
pLUS4	Derivative of pLUS2 with T substitutions at +31 and +32 in the <i>aprE</i> leader	This work
pLUS5a	Derivative of pMD433; Δ <i>amyE</i> :: <i>amyE</i> promoter– <i>amyE</i> leader– <i>lacZ</i> fusion	This work
pLUS5b	Derivative of pDG1664; Δ <i>thrC</i> :: <i>amyE</i> promoter– <i>amyE</i> leader– <i>lacZ</i> fusion	This work
pLUS6a	Derivative of pMD433; Δ <i>amyE</i> :: <i>glpD</i> promoter– <i>amyE</i> leader– <i>lacZ</i> fusion	This work
pLUS6b	Derivative of pDG1664; Δ <i>thrC</i> :: <i>glpD</i> promoter– <i>amyE</i> leader– <i>lacZ</i> fusion	This work

findings indicated the presence of a specific control mechanism for decay of *aprE* mRNA related to growth phase and perhaps operating also on mRNA for other extracellular enzymes whose synthesis is induced in stationary-phase cells.

In the present work we re-examined the results of Resnekov *et al.* (1990) with the primary aim of identifying a possible control mechanism for growth-stage-dependent differential rate of decay of *aprE* mRNA. However, we found that *aprE* mRNA has the same extreme stability in both stationary-phase and growing cells. The apparent growth-stage-dependent stability of *aprE* mRNA can be fully explained by the techniques

used by Resnekov *et al.* (1990). Our experiments also demonstrate that the determinant(s) for the extreme stability of *aprE* mRNA is contained within the leader.

METHODS

Bacteria, plasmids and primers. Bacteria and plasmids are listed in Table 1 and primers in Table 2.

Growth of bacteria. Bacteria were kept on TBAB plates. *E. coli* was grown in LB. *B. subtilis* was grown in NSMP (Fortnagel & Freese, 1968) at 37 °C on a rotary shaker at 200 r.p.m. Antibiotics were added to the following concentrations: chloramphenicol, 5 mg l⁻¹; erythromycin,

Table 2. Primers

Name	Sequence (5' to 3')
aprEBam1	GTT GGA TCC AGT CTC TAC GGA AAT AGC GAG
aprEBam2	CAA GGA TCC GAT CCA CAA TTT TTT GCT TCTC
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
aprEdel25	GGT ATA ATG GTT ACA CTC TGA ATT TT
aprEdel25inv	AAA ATT CAG AGT GTA ACC ATT ATA CC
aprEsubTT	AGT CTA CTC TTT ATT TTT TTA AAA GG
aprEsubTTinv	CCT TTT AAA AAA ATA AAG AGT AGA CT
amyE1	ATG TTT GCA AAA CGA TTC AAA ACC
amyEseq	CGA CGG TGC TGT AAG CTC ATT CGA
lacZ60	CGC CAG CTG GCG AAA GGG GG
amyEBam1	GAG GGA TCC GTT CAC AGT TTC GGG
amyEBam2	GGT TTT GGA TCC TTT TGC AAA CAT TC
amyEHindIII	GCG AAG CTT ATC CGT TCA CAG TTT CGG G
amyEconst	TTG GAT CCT TTT AAA TAA AGT AAT ACT ATG GTA TAA TGG TTA CAA GCG TTA ACA AAA TTC TCC AGT CTT CAC ATC GG
amyEconstS	GCA GAA GCT TTT AAA TAA AGT AAT ACT ATGG
lacZHindIII	GAA TAA AGC TTA AAG GTC ATT GTT GAC G
glpDBam1	CTG TGG GAT CCT GGA TCC TGG AAG GAC C
lacZseq	GTT TTC CCA GTC ACG ACG TTG

Fig. 1. Schematic representation of the inserts of *B. subtilis* LUS1 and LUS2. The *aprE* promoter and leader and part of the *aprE* coding region from BR95 were amplified by PCR and cloned in-frame with *lacZ* in pMD432. The *aprE* promoter–*aprE* leader–*lacZ* fusion was integrated into the chromosome of BR95 at the *amyE* gene to give LUS1. In LUS2, the *aprE* promoter was substituted with the *glpD* promoter. +1 indicates the transcription start point. The thick lines below the constructs illustrate the probes used to detect *aprE* and *lacZ* mRNAs, respectively.

0.5 mg l⁻¹; lincomycin, 12.5 mg l⁻¹; ampicillin, 50 mg l⁻¹; rifampicin, 100 mg l⁻¹.

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).

Enzyme activities. β -Galactosidase activity was detected on TBAB plates containing 40 mg X-Gal l⁻¹. β -Galactosidase activity in liquid cultures was assayed according to Miller (1972) as described by Glatz *et al.* (1998). Amylase activity was detected on TBAB plates containing 1.5% starch. After

incubation for 2 d, the plates were sprayed with an iodine solution (1 g iodine and 2 g potassium iodide in 300 ml distilled water). A clear halo is observed around amylase-positive colonies.

Construction of strains. A sequence containing the *aprE* promoter, leader sequence and the first 8 codons of the *aprE* gene, from -104 to +82 (Ferrari *et al.*, 1988) was amplified from chromosomal DNA of *B. subtilis* BR95 with PCR using primers aprEBam1 and aprEBam2. The fragment was cleaved at both ends by restriction enzyme *Bam*HI and ligated into the *Bam*HI site of the *B. subtilis* integration plasmid pMD432. Competent *E. coli* XL-1 Blue was transformed with the ligate with selection for ampicillin resistance. Transformants producing β -galactosidase were identified on X-Gal plates, and from one of these, pLUS1 was purified and the inserted fragment was sequenced. pLUS1 was then used to transform *B. subtilis* BR95, with selection for chloramphenicol resistance. Transformants producing β -galactosidase and lacking amylase activity were isolated. One of these was kept and the strain was named LUS1 (Fig. 1).

From plasmid pLUS1, the region containing the transcribed *aprE* part (+1 to +82) was amplified with PCR using primers aprEconst and aprEBam2. aprEconst substitutes the *aprE* promoter with the *glpD* promoter (-40 to -1) (Holmberg & Rutberg, 1992). After cleavage with *Bam*HI, the amplified fragment was ligated to *Bam*HI-cleaved pMD432. Competent *E. coli* XL-1 Blue was transformed with the ligate, with selection for ampicillin resistance. Transformants producing β -galactosidase were identified on X-Gal plates and from one of these, pLUS2 was purified and the inserted fragment was sequenced. pLUS2 was then used to transform *B. subtilis* BR95 and transformants were isolated as described above. The resultant strain was named LUS2 (Fig. 1).

Plasmid pLUS2 was used to construct two additional strains with modifications in the *aprE* leader sequence by running

PCRs with modified primers. A first PCR was run with primers aprEconstS and aprEdel25inv in one mix and aprEdel25 and aprEBam2 in another. aprEdel25inv and aprEdel25 are complementary to each other and introduce a deletion of 25 nt. The two fragments obtained were mixed and a second PCR was run with primers aprEconstS and aprEBam2. The fragment obtained was ligated to *Bam*HI-cleaved pMD432 and *E. coli* XL-1 Blue was transformed with the ligate. Ampicillin-resistant transformants were selected and pLUS3 was isolated from one of these. pLUS4 was obtained analogously but with the primers aprEdel25inv and aprEdel25 replaced with the primers aprEsubTTinv and aprEsubTT. These primers introduce T substitutions at +31 and +32. pLUS3 and pLUS4 were used to transform *B. subtilis* BR95, with selection for chloramphenicol resistance. Transformants were isolated and the resultant strains were named LUS3 and LUS4.

A sequence containing the *amyE* promoter, leader and part of the coding region, from -100 to +134 (Nicholson & Chambliss, 1986), was amplified by PCR with primers amyEBam1 and amyEBam2. The fragment was cleaved with *Bam*HI and ligated to *Bam*HI-cleaved pMD433. *E. coli* XL-1 Blue was transformed with the ligate, selecting for ampicillin resistance. Plasmid pLUS5a was isolated from one of the transformants. From pLUS5a, the region containing the transcribed *amyE* part (+1 to +134) was amplified with PCR using primers amyEconst and amyEBam2. amyEconst substitutes the *amyE* promoter with the *glpD* promoter (-40 to -1) (Holmberg & Rutberg, 1992). After cleavage with *Bam*HI, the amplified fragment was ligated to *Bam*HI-cleaved pMD433. Competent *E. coli* XL-1 Blue was transformed with the ligate, with selection for ampicillin resistance. Transformants producing β -galactosidase were identified on X-Gal plates and from one of these, pLUS6a was purified.

pLUS5a and pLUS6a were used as templates for PCRs with primer pairs amyEHindIII/lacZHindIII and amyEconstS/lacZHindIII, respectively. After cleavage with *Hind*III, the fragments were ligated to *Hind*III-cleaved pDG1664. *E. coli* XL-1 Blue was transformed with the ligates, selecting for ampicillin resistance. pLUS5b and pLUS6b were isolated and used to transform *B. subtilis* BR95. Transformants resistant to erythromycin and lincomycin (MLS^R) were selected and from each transformation a transformant that produced β -galactosidase and required threonine for growth was kept. The resultant strains were named LUS5 and LUS6, respectively.

RNA techniques. Total RNA was extracted from *B. subtilis* as described by Resnekov *et al.* (1990). For measuring mRNA half-lives, rifampicin (100 mg l⁻¹) was added and samples were then removed at intervals for extraction of total RNA. Electrophoresis of RNA for Northern blots was done as described by Thomas (1980), and the RNA was then blotted onto Hybond-N filters (Amersham). Twenty micrograms of RNA was added to each well unless otherwise indicated. Single-stranded (ss) DNA probes were generated as described before (Persson *et al.*, 2000) with the following primers: lacZ60 for *lacZ* constructs, aprEBam2 for *aprE*, and amyEseq for *amyE*. To generate templates for the ssPCR, specific fragments were amplified with PCR using the following primers and templates: glpDBam1 and lacZ60 with pLUM1041, aprEsubTT and aprEBam2 with pLUS1, and amyE1 and amyEseq with chromosomal *B. subtilis* DNA. The glpDBam1-lacZ60 fragment was cleaved with *Bam*HI and the *lacZ* part was isolated prior to the ssPCR. After hybridization, the radioactivity of the bands was quantified with a Phosphor-Imager (Molecular Dynamics). 23S rRNA and 16S rRNA were used as size markers. Primer extension analysis was performed

according to the method of Ayer & Dynan (1988). The primer used was lacZseq.

RESULTS

Construction and transcriptional control of *aprE-lacZ* fusions

A fusion was made between the *aprE* promoter, leader and the first eight codons of *aprE* (-104 to +82) (Park *et al.*, 1989) and the coding region of *E. coli lacZ* (Fig. 1). The fusion was inserted into the *amyE* locus of *B. subtilis* BR95. The resulting strain was named LUS1. A second fusion was made in which the *aprE* promoter region was substituted with the constitutive *B. subtilis glpD* promoter (-40 to -1) (Fig. 1). The fusion was inserted into the *amyE* locus in *B. subtilis* BR95 to give strain LUS2. The identity of the fusions was verified by DNA sequencing and the transcriptional start site (+1) was verified by primer extension experiments.

LUS1 and LUS2 were grown as described in Methods. At different times, samples were taken for measurement of OD₆₀₀ and β -galactosidase activity. In LUS1, β -galactosidase activity was detected only after the cells had entered stationary phase, whereas in LUS2, β -galactosidase was produced throughout exponential growth (Fig. 2). These results are in good agreement with the fact that a major control of *aprE* expression occurs at initiation of transcription through the AbrB/SpoOA switch (Olmos *et al.*, 1996).

The *aprE* leader is a determinant of extreme mRNA stability

LUS1 was grown to $t = 1.5$, i.e. for 1.5 h past the entry into stationary phase, at which time rifampicin (100 mg l⁻¹) was added to the culture. RNA was extracted at different times thereafter. The relative amounts of *aprE* and *aprE-lacZ* transcripts were de-

Fig. 2. β -Galactosidase activities of LUS1 (◆) and LUS2 (▲). The bacteria were grown in NSMP at 37 °C on a rotary shaker at 200 r.p.m. and β -galactosidase activities were measured at intervals. $t = 0$ is the time of entry into stationary phase. The results are from three individual experiments.

Fig. 3. Northern blot analysis of (a) the native *aprE* transcript of LUS1; (b) the *aprE* promoter-*aprE* leader-*lacZ* transcript of LUS1; (c) the *glpD* promoter-*aprE* leader-*lacZ* transcript of LUS2. The bacteria were grown to $t = 1.5$ and RNA was extracted at 0 min and at 7.5, 15 and 30 min after the addition of rifampicin. The experiments were repeated at least three times with similar results.

Fig. 4. Predicted secondary structures of the *aprE* leader in (a) LUS2, (b) LUS4, and (c) LUS3. In (a), the substitution of 2 nt in LUS4 is marked with arrows and the deleted 25 nt in LUS3 are bold-faced. The boxed nucleotides represent the RBS. The free energies of the secondary structures are for (a) -44.8 kJ (-37.7 kJ for the first and -7.1 kJ for the second stem-loop); (b) -65.3 kJ; (c) -33.6 kJ (<http://mfold.wustl.edu/~folder/rna/form1.cgi>).

terminated in Northern blots using probes specific for *aprE* and *lacZ*. The half-lives of the two transcripts were similar and at least 25 min (Fig. 3a, b). These results indicate that the *aprE* leader determines the stability of both transcripts and that sequences downstream of +82 in the *aprE* gene have little effect on mRNA stability. The rate of decay of the above two transcripts was also determined by primer extension analysis using a primer specific for the *lacZ* part of the *aprE-lacZ* transcript. The results of these experiments were essentially identical to those obtained with Northern blots (data not shown).

The decay of the *aprE-lacZ* transcript in stationary-phase cells was next measured in LUS2, in which the *aprE* promoter is replaced by the *glpD* promoter. Again, a half-life longer than 25 min was observed, indicating

that the rate of degradation of the *aprE* transcript is not affected by sequences upstream of *aprE* +1 (Fig. 3c).

A truncated transcript of about 700 nt was seen in the Northern blots for strains LUS1 and LUS2 (Fig. 3b, c). This represents the 5' part of the full-length transcript and has been found also in other types of transcriptional-translational fusions with *lacZ* in *B. subtilis* (Persson *et al.*, 2000). The truncated transcript decayed at the same rate as the full-length transcript, which further supports the notion that the *aprE* leader is the major stability determinant also in the fusion transcript.

To examine how changes in the *aprE* leader transcript would affect mRNA stability, two modifications were introduced into the *aprE* leader of the *lacZ* fusion in LUS2. These were chosen so as to significantly alter the secondary structure of the leader (Fig. 4). Prediction of

Fig. 5. Half-lives calculated from Northern blots of *aprE-lacZ* fusion transcripts from LUS2 (♦), LUS3 (▲) and LUS4 (■). The bacteria were grown to $t = 1.5$ and RNA was extracted at 0 min and at different times after the addition of rifampicin. The experiments were repeated at least twice with similar results.

Fig. 6. Northern blots showing steady-state levels of the *aprE* promoter-*aprE* leader-*lacZ* transcript of LUS1 after a shift-up. The bacteria were grown to $t = 1.5$ and were then diluted into fresh, prewarmed medium to OD_{600} 0.5. Samples were taken at 0 min and at 7.5, 15 and 30 min after dilution and RNA was extracted. The experiment was repeated twice with similar results.

secondary structures is very uncertain. Alternative structures are often suggested with similar free energies. One modification was a deletion of nucleotides +1 to +25, resulting in strain LUS3. In the second modification, a G and an A residue, at positions +31 and +32, respectively, were exchanged for two T residues, resulting in strain LUS4. According to computer-predicted folding (Zuker, 1989), the RBS of both LUS3 and LUS4 leader is contained within a strong stem-loop structure (Fig. 4b, c). In LUS3, the stem-loop is located at the very 5' end and in LUS4, 22 unpaired bases precede the stem-loop.

LUS3 and LUS4 were grown to $t = 1.5$, at which time rifampicin was added to the cultures. RNA was ex-

Fig. 7. An agarose ethidium bromide gel, showing increase of rRNA in *B. subtilis* after a shift-up. The bacteria were grown to $t = 1.5$ and were then diluted into fresh prewarmed medium to OD_{600} 0.5. Samples were taken at intervals during 30 min after dilution, at which time the cells were growing exponentially. Total RNA was extracted. Each lane contained RNA from 1.25 ml bacterial culture. Bands were quantified using Kodak Digital Science 1D Image Analysis Software.

tracted at different times and Northern blots were performed. The modifications in LUS3 and LUS4 destabilized the *aprE-lacZ* transcript, giving a half-life of 5–6 min (Fig. 5).

The extreme stability of *aprE* mRNA does not change with growth stage

Resnekov *et al.* (1990) reported that when a *B. subtilis* culture in early stationary phase was diluted into fresh medium, the steady-state level of *aprE* mRNA rapidly declined. This finding was interpreted as being due to a growth-stage-related destabilization of *aprE* mRNA. We have repeated and confirmed these results (Fig. 6). However, the relative rate of synthesis of rRNA rapidly increases following dilution. In Fig. 7 it is seen that the amount of rRNA increased about threefold within 30 min after a shift-up. This is in accordance with the classical observations made with *E. coli* following a growth shift-up (Bremer & Dennis, 1996). Thus, a rapid decline of *aprE* mRNA upon dilution into fresh medium could reflect a rapid dilution of this mRNA in the total RNA pool rather than accelerated degradation. To determine the half-life of *aprE* mRNA at various times after dilution is difficult because transcription from the *aprE* promoter is rapidly shut off. However, the half-life of the *aprE-lacZ* fusion transcript produced from the constitutive *glpD* promoter can readily be determined after dilution of stationary-phase cells into fresh medium as well as in exponentially growing cells. The results of such experiments using strain LUS2 show that even 30 min after dilution into fresh medium, when growth has well resumed, the *aprE-lacZ* mRNA is still ex-

Fig. 8. Northern blot analysis of the *glpD* promoter-*aprE* leader-*lacZ* transcript of LUS2. The bacteria were grown to $t = 1.5$ and were then diluted into fresh, prewarmed medium to OD₆₀₀ 0.5. Rifampicin was added 30 min after dilution. RNA was extracted at 0 min and at 7.5, 15 and 30 min after the addition of rifampicin. The experiment was repeated three times with similar results.

tremely stable, with a half-life of at least 25 min (Fig. 8). Similarly, in an exponentially growing culture of LUS2 started from a small inoculum, *aprE-lacZ* mRNA half-life is well above 25 min (data not shown).

Half-life of *amyE* mRNA and properties of *amyE-lacZ* fusions

The *aprE* gene is one of a number of *B. subtilis* genes that code for secreted proteins which are synthesized after growth has stopped and which are under (partly) common transcriptional control (Ferrari *et al.*, 1993). Another well-studied gene in this category is *amyE*, which encodes α -amylase. The half-life of *amyE* mRNA was measured in *B. subtilis* in early stationary phase and determined to be about 5 min (Fig. 9), i.e. similar to that of bulk *B. subtilis* mRNA. To compare the properties of the fusion transcripts with those of the native *amyE* transcript, a fusion between the *amyE* promoter region, the *amyE* leader and *lacZ* was made analogously to the previously described fusion involving *aprE*. The fusion was inserted into the *B. subtilis thrC* gene to give strain LUS5. LUS5 was grown in NSMP and at different points, samples were taken for determination of β -galactosidase activity. The activity was very low during growth and increased fivefold as the cells entered stationary phase (data not shown). These results confirm that expression of the *amyE-lacZ* fusion is under the same temporal control as the *amyE* gene itself.

To measure the half-life of the *amyE-lacZ* fusion transcript, we constructed LUS6, in which the tightly controlled *amyE* promoter is replaced by the constitutive *glpD* promoter. LUS6 was grown in NSMP to early stationary phase, at which time rifampicin was added to

Fig. 9. Half-lives calculated from Northern blot analysis of the native *amyE* transcript of LUS 5 (◆) and the *amyE-lacZ* transcript of LUS6 (■). The bacteria were grown to $t = 1.5$ and RNA was extracted at 0 min and at 4, 8 and 16 min after the addition of rifampicin. The experiments were repeated twice with similar results.

the culture. Total RNA was extracted at different times and the relative amounts of *amyE-lacZ* transcript were determined in Northern blots using a probe specific for *lacZ*. The half-life of the *amyE-lacZ* fusion transcript was similar to that of the native *amyE* transcript, i.e. about 5 min (Fig. 9). This demonstrates that the *amyE* leader is a major determinant of *amyE* mRNA stability and also shows the general validity of using fusion transcripts for comparisons of rates of mRNA decay.

DISCUSSION

B. subtilis aprE mRNA has been shown previously to be extremely stable in stationary-phase cells, with a half-life of at least 25 min (Resnekov *et al.*, 1990); the present work confirms this finding. Furthermore, in that earlier paper, experiments were presented which were interpreted to show that in growing cells, the rate of degradation of *aprE* mRNA was increased four- to fivefold, indicating that the stability of this mRNA was regulated by growth stage. In growing cells, the *aprE* promoter is effectively shut off, which complicates measurements of the decay of the *aprE* transcript in a growth-shift experiment. To circumvent this problem, decay of *aprE* mRNA was measured as decay of the steady-state amount of the transcript at various times after a shift from stationary phase to growth.

In the present work we have shown that the determinant(s) for the extreme stability of *aprE* mRNA is located in the 5' leader. A transcript of a transcriptional-translational fusion between the *aprE* leader and *E. coli lacZ* was found to have the same stability as the native *aprE* transcript. Furthermore, substituting the *aprE* promoter with the constitutive *B. subtilis glpD* promoter had no effect on the rate of decay of the fusion transcript. By using the *glpD* promoter one can directly measure the rate of decay of the *aprE-lacZ* fusion transcript (or the *aprE* transcript) in growing and stationary-phase cells.

The results of such measurements show that the half-life of the *aprE-lacZ* fusion transcript is at least 25 min under all conditions tested. Thus, contrary to previous suggestions (Resnekov *et al.*, 1990) there is no growth-stage-related control of *aprE* mRNA stability. The increased rate of degradation of *aprE* mRNA on shift-up observed by Resnekov *et al.* (1990) is spurious. The shift-up is immediately followed by a selective increase in stable RNA whereas the *aprE* promoter is turned off. In experiments of Resnekov *et al.* (1990) this leads to an apparent increase in degradation rate of *aprE* mRNA which is simply due to a dilution effect.

There are few data available which compare the stability of a specific *B. subtilis* mRNA at different growth stages, but increased stability is not a general property of stationary-phase mRNA (Melin *et al.*, 1989). In the present work we have also determined the half-life of *amyE* mRNA, which encodes another stationary-phase-specific *B. subtilis* exoenzyme, to be 5 min. This shows that extreme stability is not a general property of exoenzyme transcripts. Whatever the physiological reason for the exceptionally high stability of the *aprE* mRNA, it contributes to a high level of production of subtilisin in the 'stationary-phase window' where the *aprE* promoter is active.

Very few bacterial transcripts of extreme stability have been characterized, *ermC* and *gsiB* being two of the best-known examples in *B. subtilis*. The half-life of the *ermC* transcript increases about 20-fold upon exposure of the bacteria to erythromycin (Bechhofer & Zen, 1989). Binding of the drug to ribosomes translating a short ORF preceding the coding region for the *ErmC* protein stalls the ribosomes at this ORF. The stalling protects the whole *ermC* transcript from degradation, possibly by shielding a nuclease-sensitive site and/or affecting the secondary structure of the transcript. The *gsiB* transcript, which is produced from a *SigB*-dependent promoter and encodes a stress-related protein, has a half-life of at least 20 min (Jurgen *et al.*, 1998). The *gsiB* gene has a very strong RBS with an optimal spacing to the AUG start codon. Mutations that weaken the *gsiB* RBS lead to a decrease in the half-life of the transcript, suggesting that a major factor in determining the *gsiB* mRNA half-life is the interaction between the RBS and 16S rRNA. However, RBS mutations also lead to some changes in the predicted secondary structure of the *gsiB* mRNA leader sequence, which complicates the interpretation of these experiments.

The free energy of binding of the RBS and the 3' end of 16S rRNA is very similar for *gsiB*, *aprE* and *amyE* (Tinoco *et al.*, 1973) and yet the *amyE* transcript decays at least five times faster than those of the other two genes. It should be noted that the start codon is AUG for *gsiB* and *amyE* but GUG for *aprE*. Thus, a model suggesting the RBS–16S rRNA interaction to be a major determinant for mRNA stability is hardly of general validity. However, the interaction between 16S rRNA and the mRNA leader in forming the initiation complex is a function not only of base complementarity but also

of the secondary structure of the leader (Yamanaka *et al.*, 1999). The favoured predicted secondary structure of the wild-type *aprE* leader is a stem-loop at the very 5' end with the RBS contained in a region with weak interactions (Fig. 4a). The computer prediction also suggests an alternative structure with similar free energy. However, the formation of this structure involves opening the already folded stem-loops shown in Fig. 4(a). That makes this alternative structure less likely. Changing two bases in the leader (G31 and A32 both changed to T) leads to a large structural change with a perfect stem-loop containing the RBS and preceded by 22 unpaired bases (Fig. 4b). This transcript has a half-life of about 5 min. The same stem-loop was obtained by deleting 25 nt in the leader (Fig. 4c). Also this modification led to a major destabilization of the transcript. Deleting a single-stranded 5' end is known to stabilize some *E. coli* transcripts (Emory *et al.*, 1992).

Clearly, the secondary structure of the *aprE* transcript is important for the extreme stability of *aprE* mRNA. This may simply depend on occlusion of RNase-sensitive sites, but other factors may also be involved, e.g. specific (temporary or permanent) binding of proteins to the transcript. This is a known mechanism for stabilizing or destabilizing transcripts. For example, *B. subtilis glpD* mRNA can be stabilized by the GlpP antiterminator protein (Glatz *et al.*, 1996) and in *E. coli*, the *ompA* and *glgC* transcripts are destabilized by the Hfq and CsrA proteins, respectively (Vytvytska *et al.*, 1998, Liu & Romeo, 1997). Finally, we wish to point out that there are striking sequence similarities between the sequence around the *aprE* RBS and that of a polypurine-rich sequence in bacteriophage SP82 which functions as a 5' stabilizer in *B. subtilis* (Hue *et al.*, 1995).

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