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Initiation of messenger RNA decay in *Bacillus subtilis*

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INITIATION OF MESSENGER RNA DECAY IN *BACILLUS SUBTILIS*

by

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ABSTRACT

INITIATION OF MESSENGER RNA DECAY IN *BACILLUS SUBTILIS*

by

John F. Di Mari

Adviser: Professor David H. Bechhofer

The initiation of messenger RNA (mRNA) decay can play an important role in the regulation of gene expression. To date, this process is poorly understood. Here I present evidence that strongly suggests that the general mechanism for the initiation of mRNA decay in *Bacillus subtilis* is inspection at the 5' proximal region. This is demonstrated by the ability of the 5' regulatory region of the *ermC* mRNA to confer stability to diverse downstream sequences. Although initiation of mRNA decay at the 5' terminus appears to be a general mechanism, it is not obligatory since the initiation of decay can occur at other regions of the mRNA. The results of this thesis also demonstrate that segments of processed RNAs can remain stable after the initial degradative event indicating that the decay event can be blocked in *Bacillus subtilis*.

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TABLE OF CONTENTS

LIST OF FIGURES

ABBREVIATIONS

ATP	adenosine triphosphate
bp	base pair
Bs-RNase III	RNase-III-like ribonuclease in *B. subtilis*
CAT	chloramphenicol acteyltransferase
Cm	chloramphenicol
dATP	deoxyadenosine triphosphate
DNA	deoxyribonucleic acid
Em	erythromycin
methylase	23 rRNA methyltransferase
mRNA	messenger ribonucleic acid
PNPase	polynucleotide phosphorylase
RF	replicative form
RNA	ribonucleic acid
RNase	ribonuclease
SD	Shine-Dalgarno sequence
SD1	Shine-Dalgarno sequence of the *ermC* leader peptide
SD2	Shine-Dalgarno sequence of the *ermC* methylase

Chapter One

Objective and Background

Objective

1.1 Overview:

The initiation of messenger RNA (mRNA) decay is an important step in the regulation of gene expression. Much of the current understanding of mRNA decay has been gained from research using the gram negative bacterium *Escherichia coli* as a model. The experiments described in this thesis have been designed to aid in our understanding of the processes involved in the initiation of mRNA decay in the gram positive bacterium *Bacillus subtilis*.

The erythromycin (Em) resistance gene *ermC* has been selected as a model for the initiation of mRNA decay in *B. subtilis*. This gene was selected since the mRNA transcribed from this gene can be inducibly stabilized by the stalling of Em-bound ribosomes in the 5' leader region of the transcript. The observation that this mRNA was stabilized by 5' protection may indicate that initiation of mRNA decay in *B. subtilis* occurs at the 5' terminus. Alternatively, the *ermC* mRNA may be unique in that its only target site for the initiation of decay may be in the 5' leader region. The initiation of decay of other *B. subtilis* mRNAs may occur at downstream target sites.

Here I present a set of experiments designed to determine if the initiation of decay of the *ermC* mRNA must occur at the 5' terminus in *B. subtilis*. A report describing some of these experiments has been accepted for publication (Di Mari and Bechhofer,1993).

Background

1.2 Prokaryotic mRNA Decay:

The regulation of gene expression is essential for the vitality of an organism. It is imperative that an organism be capable of manipulating the level of expression of its genes so that it can adequately respond to its environment and yet conserve as much energy as possible. The levels at which an organism can maintain control of expression range from the rate of transcription to the stability of the final protein product. Intensive studies have been carried out on the mechanisms of transcription and translation and have led to a basic understanding of these processes. However, the mechanism(s) by which messenger RNAs are degraded has remained poorly defined.

The cellular concentration of functional mRNA can be an important factor in the regulation of gene expression. The level of cellular mRNA is a product of the balance of the transcription rate and the rate of mRNA degradation. The rate of degradation is limited by the rate of association of ribonucleases (RNases) with the mRNA. This association can be affected by mRNA secondary structure and by protection of the mRNA by ribosomes or other *trans*-acting stabilizers. Here I describe several factors which may play a role in the initiation of mRNA decay.

1.2A The Role of Ribosomes:

The regulation of mRNA decay must be a product of how a transcript interacts with its environment. This interaction would be influenced by factors that either protect the transcript or make it more accessible to the degradative machinery. One factor that may influence the accessibility of a transcript to its environment is the presence of

ribosomes and their association with a transcript. The presence of ribosomes on a transcript may act to protect the transcript by blocking endonucleolytic cleavage sites. Early studies demonstrated that a blockage of translation of the *E. coli* tryptophan (*trp*) mRNA led to an increase in the rate of decay (Morse and Yanofsky,1969). However, further studies demonstrated that this is not universally true. Mutations that altered the efficiency of translation of the *E. coli* β-galactosidase (*lacZ*) mRNA and thus reduced the number of ribosomes present on the transcript were found to have no effect on the rate of mRNA decay (Stanssens *et al.*, 1986). An examination of the degradation of the mRNA for the major outer membrane protein of *Escherichia coli* (*ompA*) lead to the discovery that the 5' untranslated region of the mRNA was the most stable portion of the transcript (von Gabain *et. al.*, 1983). Analysis of an *ermC* mutant in *B. subtilis* in which a lengthy portion of the mRNA remains untranslated demonstrated no difference in the rate of decay compared to fully translated mRNA (Bechhofer and Dubnau,1987). Therefore, the role that ribosomes play as a general contributor to the rate of mRNA decay is questionable.

1.2B 3'-To-5' Exonucleolytic Decay:

The degradation of RNA to mononucleotides in *E. coli* occurs via processive decay in the 3'-to-5' direction. The enzymes responsible for this degradation are ribonuclease II (RNase II) and polynucleotide phosphorylase (PNPase). These enzymes have been characterized as 3'-to-5' exoribonucleases whose functions are redundant since bacterial strains carrying nonfunctional mutations in either of these enzymes exhibit

normal patterns of mRNA decay (Donovan and Kushner,1983). Although the functions of these proteins are the same, the mechanisms by which they operate are not. RNase II hydrolyzes RNA transcripts into ribonucleotide monophosphates while PNPase works phosphorolytically converting transcripts into ribonucleotide diphosphates. Strains that are devoid of PNPase activity and that express a temperature sensitive RNase II accumulate message degradation intermediates at the non-permissive temperature (Donovan and Kushner,1986) and show an increase in specific message half-lives (Mackie,1989;Plamann and Stauffer,1990). These findings, coupled with the fact that a simultaneous absence of both of these proteins is lethal, demonstrate the importance of these two proteins in mRNA decay in *E. coli* (Belasco and Higgins,1988).

The ability of 3' proximal secondary structure to impede the procession of the 3'-to-5' exoribonucleases has been suggested by several studies. The stability of the mRNA encoding the *E. coli glyA* gene product, SHMT, which converts serine to glycine, is dependent on two stem-loop structures and a rho-dependent transcriptional terminator in its 3' proximal region (Plamann and Stauffer,1990). Removal of these structures led to a rapid degradation of this mRNA. In the maturation of the tryptophan (*trp*) operon mRNA, the processing by a 3'-to-5' exoribonuclease appears to be blocked by a stem-loop structure at the 3' proximal region (Mott *et al.*,1985). While several studies have demonstrated the ability of 3' proximal stem-loop structures to inhibit the procession of 3'-to-5" exoribonucleases *in vivo, in vitro* studies suggest that these stem-loop structures alone do not provide a sufficient block to the progression of exoribonucleases (McLaren *et al.*,1991). Analysis of the half-lives of transcripts demonstrated that while a 3' stem-loop structure could provide a 15 minute half-life *in*

vivo, the same structure produced a half-life of less than 3 minutes *in vitro*. Further analysis demonstrated that the stability of the stem-loop (measured in kcal/mol) was not important and that the rate of decay was not a function of the rate of stem-loop melting. The authors concluded that the difference in the observed half-lives of the stem-loop structures *in vivo* versus *in vitro* was due to additional factors that are involved in stabilizing mRNA *in vivo*. One possible factor is a stem-loop binding protein that provides protection from exoribonuclease attack. The degradation of such a protected transcript would not be initiated by exoribonucleolytic attack, but rather by some upstream endoribonucleolytic event.

1.2C 5'-To-3' Decay:

The degradation of mRNA in a 5'-to-3' direction is a rather attractive model. This mode of decay would be the most energetically favorable since it would prevent unnecessary translation. Degradation from the 5' terminus would allow for the removal of a ribosome binding site and therefore prevent the translation of an unneeded protein. If mRNA decay were to proceed in the 3'-to-5' direction at the same time that translation was occurring in the 5'-to-3' direction of the same mRNA, this would lead to truncated protein formation since 3' proximal codons would be missing. The translation of incomplete proteins would not be energetically favorable. The flow of RNases in the 5'-to-3' direction following behind the last translating ribosome would seem a more logical occurrence than the flow of RNases upstream against the procession of ribosomes. Therefore a 5'-to-3' directionality of decay would seem most logical.

Early studies to determine the direction of decay of mRNA in *E. coli* were performed on mRNA from the *trp* operon (Morse *et al.*,1969). The results of these

experiments demonstrated that 5' proximal regions decayed before the distal regions of mRNA. Studies of the decay of mRNA of the *E. coli* galactose (*gal*) and lactose (*lac*) operons demonstrated that degradation did not occur via a 5'-to-3' exoribonuclease (Achord and Kennell,1974;Blundell and Kennell,1974). However, experiments performed on the directionality of decay of the β–galactosidase (*lacZ*) message demonstrated that the *lacZ* mRNA was degraded by a net directional wave in the 5'-to-3' direction (Cannistraro and Kennell, 1985).

Degradation in a 5'-to-3' direction could be explained by a 5'-to-3' exoribonuclease which proceeds along the transcript in a processive fashion. To date, no 5'-to-3' exoribonuclease has been isolated from *E. coli*. If no 5'-to-3' exoribonuclease exists how then can one explain this directionality of decay? The processive decay in a 5'-to-3' direction could be explained by a series of endonucleolytic cleavages in a 5'-to-3' direction coupled with a 3'-to-5' exonuclease activity for mRNA turnover. Several models have been postulated to explain this mode of decay. In Figure1A a model is given which has been used to explain the decay of *lac* mRNA (Cannistraro *et al.*,1986). Here, a wave of translating ribosomes blocks the accessibility of endonucleolytic cleavage sites that would be randomly cleaved on a naked mRNA. The initial cleavage event occurs in or near the ribosome binding site in the 5' proximal region of the mRNA. Such a cleavage would prevent the loading of additional ribosomes and therefore remove the protection conferred to the message by the wave of translating ribosomes. The message would then be degraded by successive endonucleolytic attacks which would follow the 5'- most translating ribosome. While this model may hold true in

some cases (*e.g.*, *lac* mRNA decay) it is not a universal truth. As was mentioned above (1.2A), the rate of decay of poorly translated or untranslated mRNA sequences were not always found to decay more rapidly than translated sequences. These results would not agree with this model of decay.

An alternate model is proposed in Figure 1B (Belasco and Higgins,1988). In this model a "5'-binding" endoribonuclease associates with the message at or near the 5' terminus of the transcript. This endoribonuclease then migrates along the transcript or loops out searching for its target sites. Once cleavage has occurred, the cleaved fragments are converted to mononucleotides by a 3'-to-5' exonuclease. The rate of decay would be limited in this model by the availability of the 5' region of the mRNA to the endoribonuclease and the rate of association of this enzyme with the 5' terminus. Thus the rate of decay would be affected by 5' secondary structure and factors which may associate with the 5' proximal region of the mRNA.

The significance of the 5' proximal sequence to the decay rate of mRNA has been reported. In exponentially growing *E. coli* cells the *ompA* transcript was found to have a half-life of 18 minutes, which is determined by its 5' proximal region (Emory and Belasco,1990). In contrast, the β-lactamase (*bla*) mRNA has a half-life of 3 minutes. If the 5' stabilizing region of *ompA* is fused to the coding region of *bla*, the half-life of the transcript is 3- to 5-fold higher than that of the wild-type *bla* mRNA (Belasco *et al.*,1986). Removal of the 5' untranslated region of this transcript, which can form a stem-loop structure, led to a decrease in half-life to 3-4 minutes (Chen *et al.*,1991). Phylogenetic comparisons of the 5' untranslated regions of *ompA* transcripts of *E. coli*,

Serratia marsescens and *Enterobacter aerogenes* (all gram negative organisms), revealed that although the sequence of this region was diverse among these organisms, the structure of the 5' untranslated regions was conserved (Chen *et al.*,1991). Mutational studies showed that the structure and not the sequence of the 5' proximal RNA was critical for stability (Emory *et al.*,1992). Experiments revealed that two unpaired bases at the 5' terminus could be tolerated without affecting the rate of decay, but an unpaired sequence of 5 nucleotides or more at the 5' terminus led to an accelerated rate of decay. This data demonstrates the importance of 5' terminal structure on the rate of decay. The significance of this structure may be to block an as yet unidentified 5'-to-3' exoribonuclease or a 5' dependent endoribonuclease that cleaves internally but must first interact with an accessible 5' terminus. Inactivation of the RNase E protein led to an increase in *ompA* mRNA half-life and this protein may be the putative 5'-dependent endoribonuclease (Lundberg *et al.*,1990).

The initiation of decay at the 5' proximal region of an mRNA in *E. coli* has been demonstrated. However, the conversion of mRNA to mononucleotides has only been demonstrated in the 3'-to-5' direction. This results may appear contradictory, however, one should note that the initiation of decay and the directionality of decay are not the same thing. The association of an RNase with the 5' terminus of the mRNA followed by the initial cleavage event that can occur anywhere on the mRNA is the initiation event. This event would be the rate limiting step in mRNA decay. The conversion of the mRNA into mononucleotides ensues this event and occurs in the 3'-to-5' direction.

1.2D Endoribonucleases:

The first endoribonuclease to be isolated from *E. coli* was RNase III, the protein product of the *rnc* gene, which was found to cleave double-stranded RNA *in vitro* (Robertson *et al.*,1968). This endoribonuclease functions mainly in the maturation of the 30S RNA encoded by the *E. coli* ribosomal operons but also plays a role in mRNA metabolism (Deutscher,1988). This enzyme has been found to cleave ribosomal RNA (rRNA) in a region that contains two nearly perfect RNA-RNA duplexes that are over 20 base pairs long (Young and Steitz,1978). Other RNase III substrates contain two small stems of 7-10 base pairs in length separated by a "bubble" with 4 unpaired bases on one side and 5 unpaired bases on the other (Robertson,1982). Analysis of known RNase III cleavage sites demonstrated that this enzyme did not recognize a particular sequence but appeared to have an affinity for stem-loop structures formed by RNA duplexes. Analysis of RNase III cleavage demonstrated that this enzyme may cleave a substrate at one or two sites but what the determinants of the mode of cleavage are remain unknown.

The effect of RNase III cleavage on an mRNA varies depending on the location of the cleavage site, the number of cleavages at that site and the remaining secondary structure of the processed mRNA. The removal of a 3' stem-loop structure by RNase III cleavage may result if RNase III cleaves on both sides of the stem-loop. Such processing may result in a destabilization of the mRNA since it could render the transcript accessible to 3' exonucleolytic attack. This has been demonstrated in the retroregulation of the λ *int* gene by the downstream *sib* sequence (Schmeissner *et al.*,1984). Transcription into the downstream *sib* sequence produces a target site for RNase III that is not formed if

transcription stops at the *int* terminator. During a lysogenic infection the *int* gene is down regulated due to an increase in transcription past the normal terminator producing transcripts that now contain RNase III target sites. This cleavage produces a 3' terminus that is susceptible to exonucleolytic attack which degrades the mRNA sequences of the *int* coding sequence.

Cleavage at a 3' proximal RNase III cleavage site has also been found to stabilize a transcript. Since few bacterial mRNAs are cleaved by RNase III, an artificial mRNA was used to determine the effect of RNase III cleavage on mRNA (Panayotatos and Truong,1985). A bacteriophage T7 RNase III cleavage site was fused downstream of the coding sequence of a constitutively expressed human interferon α5 gene in *E. coli*. Analysis of the mRNA transcribed from this fusion isolated from both an *rnc*$^+$ and an *rnc*$^-$ strain demonstrated that this transcript was indeed processed by RNase III. Half-life analysis of the processed versus the unprocessed mRNA revealed that the processing led to an increased half-life of 9 minutes compared to 2.5 minutes for the unprocessed mRNA. 3' terminal mapping revealed that RNase III cleavage occurred on the 3' side of the stem-loop, which left the 3' terminus of the mRNA sequestered in a stem-loop. The authors believed that this produced the increase in half-life since the mRNA would now be resistant to 3'-to-5' exonucleolytic attack.

RNase III has also been shown to cleave mRNAs in their 5' proximal regions. Such processing has been demonstrated in the mRNAs coding for RNase III itself and PNPase (Bardwell *et al.*,1989;Takata *et al.*,1989). Processing of these mRNAs led to a decrease in mRNA half-life due to the removal of 5' proximal stem-loop structures. The half-lives of the PNPase and RNase III mRNAs dropped from 8 minutes to 1.5 minutes

and 4.8 minutes to 0.75 minutes, respectively, in an *rnc*⁻ versus an *rnc*⁺ strain. The decrease in half-lives after processing may be due to an increased accessibility of the 5' terminus to ribonucleases or by RNase III aiding in the association of other ribonucleases with the transcripts.

Recently, the product of the *ams* gene (altered mRNA stability) has received a great deal of attention. It was observed initially that a strain containing a temperature-sensitive mutation in this gene demonstrated increased mRNA stability at the non-permissive temperature (Kuwano *et al.*,1977). The same effect on general message stability was observed for strains containing a mutated *rne* gene, encoding ribonuclease E (RNase E), and further studies showed that *ams* and *rne* genes map at the same locus and are the same gene (Mudd *et al.*,1990b).

RNase E was initially described as a 91 kDa ribonuclease involved in the maturation of 5S RNA (Ghora and Apirion,1978) and in the processing of bacteriophage T4 transcripts for genes *32* and *59* (Mudd *et al.*,1990a). Recent evidence has shown that this RNase is actually 114 kDA and that the initial sequencing of its open reading frame omitted two nucleotides which led the authors to believe the protein was 91 kDa (Casaregola et al.,1992). A strain containing a temperature sensitive mutant RNase E had a 5-6 fold increase in the half-life of its mRNA at the nonpermissive temperature (Ono and Kuwano,1979). Cleavage by this protein can lead either to an increase in transcript stability or an acceleration of decay (Mudd et al.,1990a; Regnier and Hajnsdorf,1991). The activity of this protein was found to be essential for the initiation of decay of the *ompA* transcript as well as for other mRNAs since removal of RNase E activity led to a stabilizing effect of these mRNAs (Lundberg et al.,1990;Ono and

Kuwano,1979). These results indicated that RNase E, the protein product of the *rne* gene, may be a factor in the initiation of mRNA in general in *E. coli*.

Analysis of the function of RNase E has demonstrated that this ribonuclease is an endoribonuclease that cleaves proximal to a stem-loop structure at a consensus sequence defined as (A/G)AUU(A/U) (Ehretsmann *et al.*,1992). Most recently, it has been demonstrated that the activity of RNase E is sensitive to 5' proximal base pairing of its target mRNA. RNA I is a 108 nucleotide RNA from plasmid pBR322 that controls plasmid copy number. This RNA was found to be a substrate for RNase E (Tomcsanyi and Apirion,1985) and has a half-life of about 2.5 minutes (Lin-Chao and Cohen,1991). Stem-loop structures were fused upstream of the 5' terminus of this RNA and RNA half-lives were determined (Bouvet and Belasco,1992). Constructs which resulted in a 5' terminus sequestered in a stem-loop structure were found to have an increased stability over the wild type RNA I transcript regardless of the positional distance from the RNase E cleavage site. However, no difference in stability was detected when an accessible 5' terminus was present upstream of the stem-loop structure. The half-life of RNA I and its derivatives that contain accessible 5' termini were found to increase 5- to 10-fold at the nonpermissive temperature in a strain containing a temperature sensitive mutant of RNase E compared to the half-lives at the permissive temperature. These results indicate that the association of this enzyme with an accessible 5' terminus of an mRNA appears to be essential for its activity.

1.2E The Regulation of Gene Expression by mRNA Decay:

The regulation of message decay as a means of determining the level of expression of a gene is becoming more apparent. The differential expression of genes transcribed as a polycistronic message demonstrates this point. In *E. coli* the *malEFG* operon has been shown to be regulated by differential decay of various regions of the mRNA (Newbury *et al.*,1987). This operon is part of the maltose/maltodextrin transport system. The abundant periplasmic protein *malE* is expressed in a 20- to 40-fold excess over the membrane- associated *malF* and *malG* proteins. This difference has been shown to be directly correlated to a difference in the cellular levels of RNA encoding these proteins. Cleavage between the *malE* and *malF* coding regions leads to a rapid decay of *malFG*, but leaves a stable *malE* transcript that terminates in a 3' stem-loop structure that form in a REP sequence (Repetitive Extragenic Pallindromic sequence).

Differential expression of various regions of a polycistronic message has also been demonstrated for other *E. coli* operons. The arsenical resistance (*ars*) operon encodes three structural genes (*arsABC*). A small amount of the *ArsB* protein is made while the *ArsA* and *ArsC* proteins are abundant. The difference in the relative amounts of these proteins has been attributed, in part, to the difference in the rates of decay (Owolabi and Rosen,1990). The full length *ars* mRNA had a half-life of 4.4 minutes. This mRNA was then processed leaving the 5' proximal sequence encoding the *arsA* protein and the distal sequence encoding the *arsC* protein, the central region encoding *arsB* protein was not detected after processing. The half-lives of the mRNA segments encoding the *arsA* and *arsC* proteins were about 10 minutes. The authors concluded that this difference in

half-lives along with differences in translation contributed to the relative amounts of these proteins. The *atp* operon of *E. coli* encodes nine genes, eight of which form the membrane-bound H^+-ATPase. The relative rate of protein synthesis was found to correlate to the stoichiometric amounts of the complex. The difference in the relative amount of protein production of these subunits was attributed in part to segmental differences in mRNA stability (McCarthy *et al.*,1991).

The regulation of gene expression by mRNA decay is not limited to the differential decay of polycistronic messages. The expression of RNase III and PNPase is controlled by autoregulation in which overproduction of the proteins leads to an increase in the degradation in their mRNAs (Bardwell *et al.*,1989;Robert-Le Meur and Portier, 1992). The decay rate of the *E. coli ompA* mRNA was found to be growth rate dependent (Nilsson *et al.*,1984). The half-life of the message was found to decrease from about 17 minutes to 4 minutes when cells were shifted from conditions of rapid cell growth to those of poor cell growth. This difference in mRNA half-life was proportional to the difference in the production of *ompA* protein. These data demonstrate that the regulation of mRNA decay rate can be a means of regulating gene expression.

1.2F mRNA Decay in Other Organisms:

Much of our understanding of the processes involved in prokaryotic gene expression are based on experimentation with *E. coli*. The same holds true for our understanding of mRNA decay in prokaryotes. The expectation that similarities between the processes involved in mRNA decay in *E. coli* and that of other bacteria is not

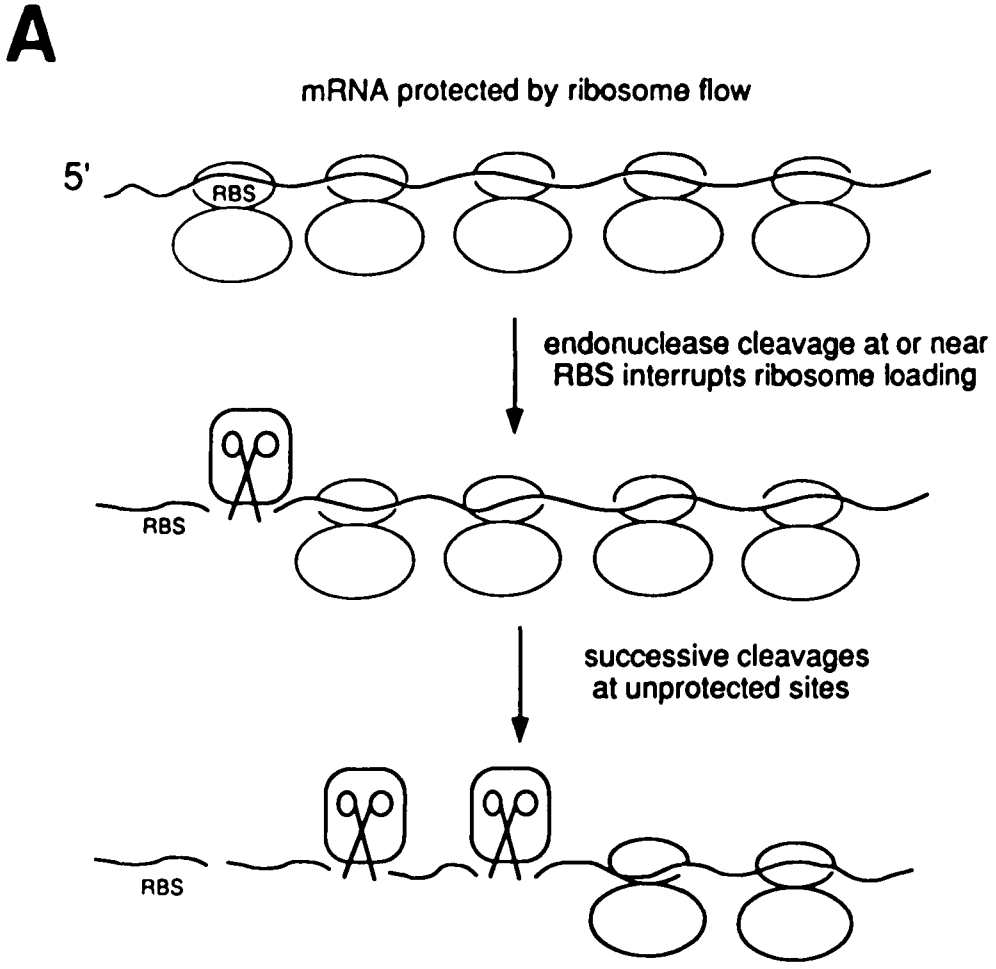
unwarranted since other molecular events have been found to be quite similar. Regulation of gene expression of polycistronic messages by differential decay has been demonstrated in other bacteria. The *gap* operon of *Zymomonas mobilis* is a bicistronic message that encodes the genes for glyceraldehyde-3-phosphate (*gap*) and phosphoglycerate kinase (*pgk*). Due to differences in their catalytic efficiency, 3- to 4-fold more *gap* protein is needed than *pgk* protein. The difference in the relative abundance of these proteins has been attributed to the differences in their mRNA stabilities. The full length bicistronic mRNA was found to have a half-life of 7 minutes while a processed transcript encoding the *gap* protein had a half-life of 16 minutes (Eddy *et al.*,1991). The differential expression of the photosynthetic *puf* operon of *Rhodobacter capsulatus* was found to be produced by segmental differences in mRNA decay (Belasco *et al.*,1985). The initial transcript encodes the genes for the *pufQBALMX* proteins. The *pufBA* proteins are produced in a 12-fold excess over the *pufLM* proteins. The full-length mRNA had a half-life of 3 minutes while a processed transcript encoding *pufBA* was found to have an 18 minute half-life. The authors concluded that the difference in the relative amount of protein was a product of the differential decay of the mRNAs.

The mechanisms involved in the degradation of mRNA in the gram positive bacterium *Bacillus subtilis* are a central concern in Dr. Bechhofer's laboratory and in this thesis. It has been demonstrated that gene expression can be regulated in *B. subtilis* by mRNA decay. The *B. subtilis* succinate dehydrogenase operon (*sdh*) is transcribed as a polycistronic message encoding cytochrome b_{558} (*sdhC*), a flavoprotein (*sdhA*) and an iron-sulfur protein (*sdhB*), in that order. The stability of this message was found to be

growth rate dependent with a decrease in message half-life from 2.6 to 0.4 minutes as the cells entered stationary phase (Melin et al.,1989). Further studies demonstrated that the regulation of decay of this mRNA was dependent on its 5' proximal sequence (Melin *et al.*,1990). These results demonstrate that a 5' proximal sequence can act as a regulator of mRNA stability in *B. subtilis* just as it can in *E. coli* (*e.g., ompA*). The mode of decay for other mRNAs in *B. subtilis* has not been reported.

Are the mechanisms of mRNA decay identical in *B. subtilis* and *E. coli*? Examination of RNA decay in *E. coli* indicated that this process was mainly hydrolytic while RNA decay in *B. subtilis* was phosphorolytic (Deutscher and Reuven,1991). In *E. coli*, 90% of the activity for the decay of mRNA to mononucleotides was attributed to the hydrolytic 3'-to-5' exoribonuclease, RNase II. This enzyme, or an equivalent thereof, is absent in *B. subtilis*, and the mode of mRNA decay is phosphorolytic in nature, primarily due to a *B. subtilis* equivalent of PNPase. These results suggest that the exact mechanisms of mRNA decay are not the same in these two organisms. Our studies of the initiation of decay in *B. subtilis* will shed light on this process in gram positive organisms and may be instructive when compared with the process in *E. coli*.

Figure 1. Models for the initiation of mRNA from the 5' region. (A) Postulated model for the initiation of *lac* mRNA in *E. coli* (Cannistraro *et al.*,1986). A high density of translating ribosomes confers resistance to the RNA by blocking endonucleolytic cleavage sites. A random cleavage event near the ribosome binding site interrupts the binding of ribosomes. A wave of cleavages in the 5'-to-3' direction occurs following the last translating ribosome. (B) The endonuclease contains a binding site (closed circle) for the 5'-end of an mRNA. After 5'-end binding, the endonuclease can migrate or loop to a downstream cleavage site (Emory *et al.*,1992).


A
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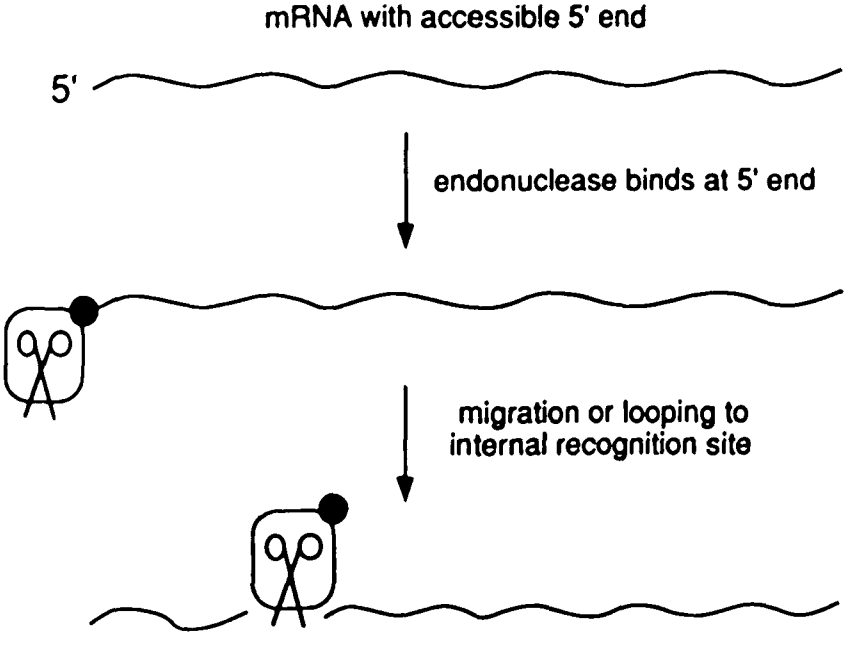
5'
RBS
endonuclease cleavage at or near
RBS interrupts ribosome loading
RBS
successive cleavages
at unprotected sites
RBS

B


mRNA with accessible 5' end
5'
endonuclease binds at 5' end
migration or looping to
internal recognition site

1.3 Regulation of *ermC*:

We have chosen to study the decay of messenger RNA encoded by the *ermC* gene in a *B. subtilis* host. The *ermC* gene was originally identified on plasmid pE194 as the component of the plasmid that conferred erythromycin (Em) resistance in *Staphylococcus aureus* (Iordanescu,1976). pE194 was later transformed into *B. subtilis* (Weisblum *et al.*,1979). A schematic of the *ermC* gene is shown in Figure 2. The *ermC* gene is transcribed as a 910 nucleotide mRNA that encodes a 19 amino acid leader peptide and a 29 kDA protein. This protein has been identified as a ribosomal RNA methyltransferase (methylase) that confers Em resistance by methylating a specific adenine residue of 23S rRNA, which thereby prevents the binding of erythromycin to the ribosome (Skinner *et al.*,1983)

The *ermC* message is transcribed from a constitutive promoter and the regulation of *ermC* gene expression was determined to be posttranscriptional (Shivakumar *et al.*,1980). Expression of *ermC* is induced in the presence of Em and the induction is due to an increase in translation (Shivakumar *et al.*,1980). In order to explain this induction, the "translational-attenuation" model has been proposed (Gryczan *et al.*,1980).

According to the translational-attenuation model, the 5' region of the *ermC* message forms a stem-loop structure, which has been verified by structural analysis (Narayanan and Dubnau,1985; Mayford and Weisblun,1985). In the uninduced conformation, the ribosome binding site (SD1) for the leader peptide is accessible to ribosomes, but the ribosome binding site (SD2) for the methylase is sequestered (Figure 3). In this conformation the methylase is only expressed at a basal level. Upon the

addition of Em, the system becomes induced. Em-bound ribosomes stall while translating the leader peptide and this stalling disrupts the message secondary structure and makes SD2 accessible for translation by ribosomes that are not bound by Em (Figure 4).

At the same time as the increase in translation, an increase in message half-life is also observed (Shivakumar et al.,1980; Bechhofer and Dubnau,1987). Northern blot analysis was performed on RNA isolated from a *B. subtilis* strain that harbored a plasmid containing the *ermC* gene. RNA was isolated at various time points after the addition of rifampicin in the presence and absence of Em. The results of this analysis are shown in Figure 5. The presence of Em had a dramatic effect on the half-life of the *ermC* message. The uninduced half-life of *ermC* mRNA was 2 minutes while the half-life of *ermC* mRNA in the induced state increased to about 40 minutes.

The observed increase in mRNA stability could be a direct effect of the increase in translation of the methylase coding sequence. During induction, the concentration of ribosomes in the body of the message increases due to the increased accessibility of the SD2 site, and this ribosome density could protect the mRNA by blocking ribonuclease target sites. To show that this was not the case, the SD2 site was made nonfunctional by mutating the sequence from AGAGGG to ACCCGG (Figure 3). Northern blot analysis of a strain harboring this mutation demonstrated that the induced stability of the *ermC* mRNA was not a result of increased translation since the half-lives of this transcript in the presence and absence of Em were the same as the wild-type mRNA (Bechhofer and Dubnau,1987).

It was demonstrated that induction of *ermC* mRNA stability is caused by the stalling of an Em-bound ribosome in the 5' leader region. The second codon of the *ermC*

leader peptide was changed from GGC to TAA so that the mRNA would contain an ochre mutation, thus preventing translation of the leader peptide. Northern blot analysis of this transcript in the presence and absence of Em revealed that the mRNA could not be stabilized. This transcript was inducibly stable when expressed in a suppressor strain indicating that the loss of induced stability was due to the absence of translation of the leader peptide and not the mutated sequence itself. Deletions were made at various points in the stem-loop sequence and the mRNA from these mutants was inducibly stable as long as the coding sequence for the leader peptide remained intact (Hue and Bechhofer,1991). Thus the induced stability of the *ermC* mRNA is not due a conformational change in the stem-loop structure leading to a loss of an RNase target site. These results indicate that the only requirement for the induction of stability of the *ermC* mRNA is the translation of the leader peptide and the stalling of Em-bound ribosomes in this region. This implies that the initiation of *ermC* mRNA decay occurs in the 5' region since protection of this region by ribosomes protects the entire mRNA.

Em-bound ribosome stalling could protect *ermC* mRNA in several ways. The stalled ribosome(s) could block a target site located in the leader peptide coding sequence that is essential for the initiation of decay. Alternatively, the initiation of decay may require an accessible 5' terminus, and ribosome stalling in the leader peptide coding sequence may protect the 5' terminus which is 40 nucleotides from the stall site. The distance from the 5' terminus of the mRNA and the leader peptide was increased by cloning DNA fragments between the *ermC* promoter and the +1 site of transcription (Bechhofer and Zen,1989). Northern blot analysis of these mRNAs revealed that these transcripts contained an unstable 5' proximal region in both the presence and absence of

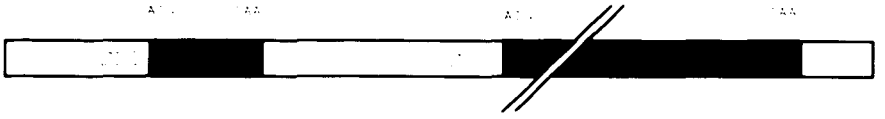
Em and sequences from the ribosome stall site and downstream were relatively stable. The authors concluded from these data that Em-bound ribosomes protect the mRNAs from degradation by either a 5'-to-3' exoribonuclease or a processive endoribonuclease that binds to the 5' terminus.

The fact that ribosome stalling in the 5' leader region of *ermC* can impart stability to the entire message implies that the remainder of the message is resistant to other nucleases. This could be due to a quality of the mRNA itself or to the fact that the initiation of mRNA decay in *B. subtilis* must occur at the 5' terminus. The following experiments were designed to address this issue. Fusions were made between the 5' leader region of *ermC* and diverse downstream sequences. Additionally, endoribonucleolytic cleavage sites were inserted into the methylase coding sequence of *ermC* to examine the potential for alternate modes of mRNA decay initiation in *B. subtilis*.

Figure 2. Schematic of the *ermC* gene. (A) Diagram of the *ermC* gene, the mRNA transcribed from it, and the translational products. The gene is represented by the rectangle. The open reading frames of the gene are represented by filled bars. The non-translated regions are represent by open bars. *ermC* mRNA and the translated products are represented by lines. The ribosome binding sites (SD) are shown, as well as the initiation and stop codons for translation. (B) Schematic of the ermC gene and the probes used for Northern analysis. The site for the initiation of transcription is shown (+1). The probes are represented by the filled bars.

A

ermC Gene



B

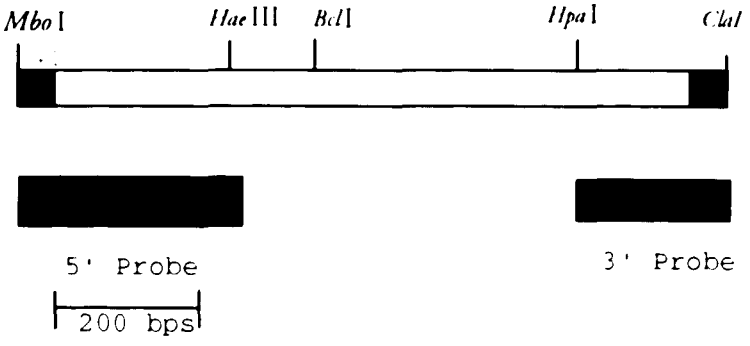


Figure 3. Uninduced structure of the 5'-regulatory region of *ermC*. Ribosome binding sites for the leader peptide (SD1) and for the methylase coding sequence (SD2) are shown. Initiation and stop codons are underlined. The mutated SD2 sequence is shown adjacent to the SD2 sequence.

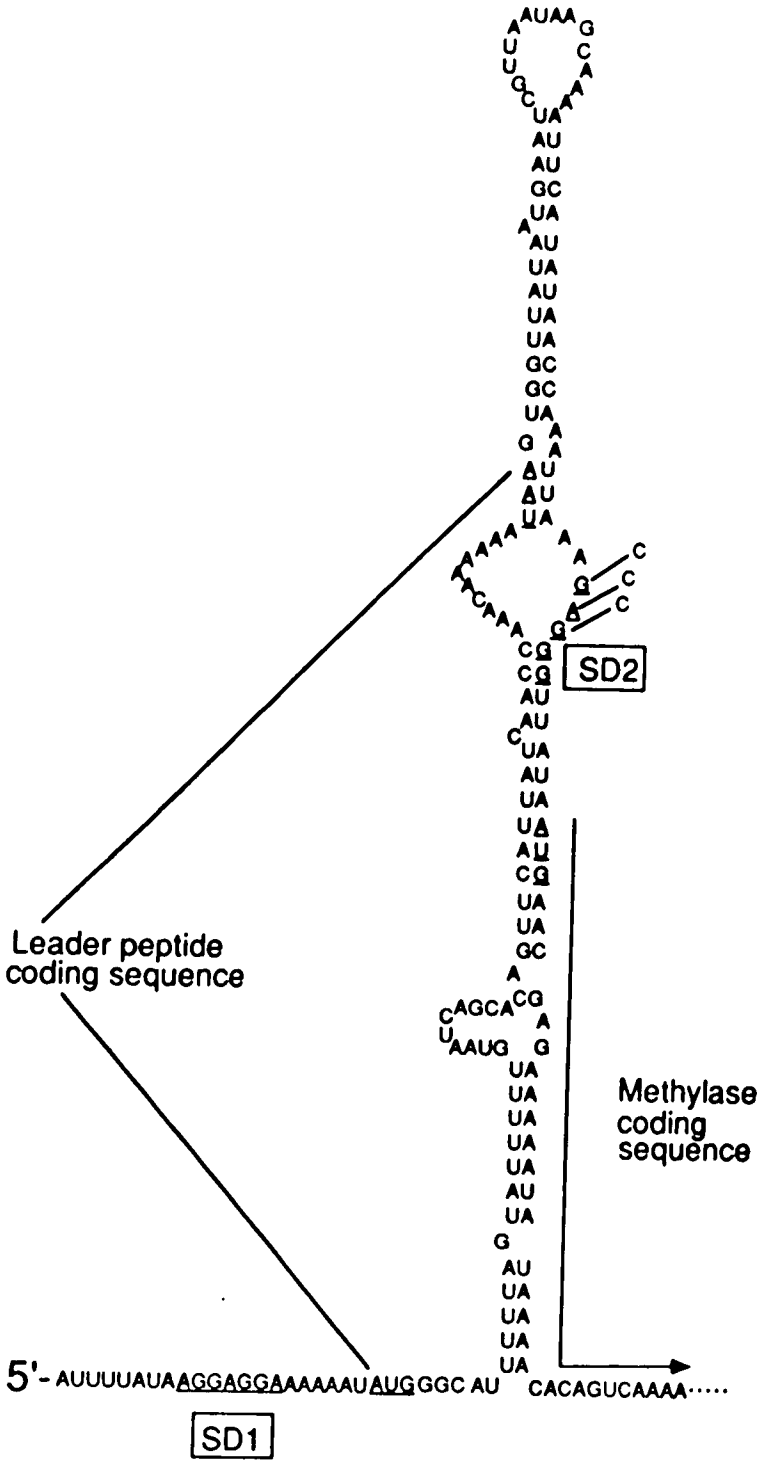

Leader peptide
coding sequence
SD2
Methylase
coding
sequence
5'- AUUUUAUAAGGAGGAAAAAAUAUGGGC AU CACAGUCAAAA·····
SD1

Figure 4. Model for the induction of the *ermC* gene. Em (Δ) binds to unmethylated ribosomes. The methylated ribosomes stall in the leader peptide sequence, causing a conformational change in the *ermC* mRNA that makes SD2 accessible to non-Em-bound ribosomes. Methylated ribosomes are indicated by a filled circle present in their icon. These ribosomes are not bound by Em and play no role in induction.

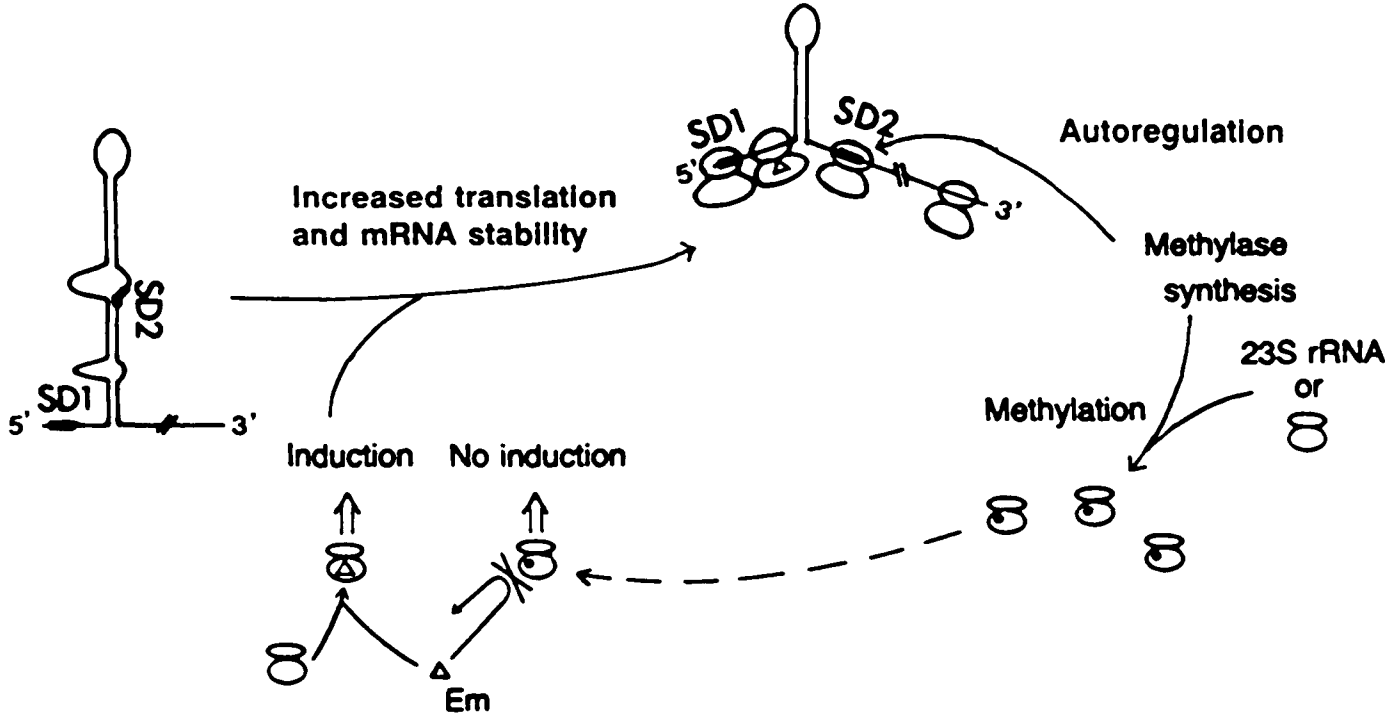
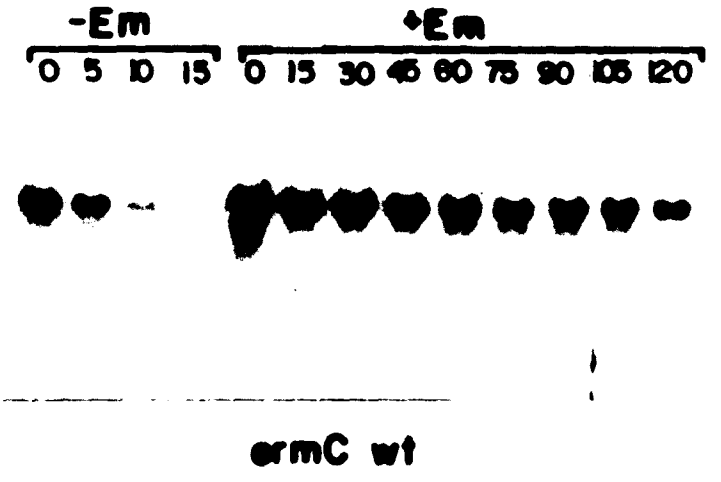

SD1
SD2
5'
3'
Increased translation
and mRNA stability
SD1
SD2
5'
3'
Autoregulation
Methylase
synthesis
23S rRNA
or
Methylation
Induction
No induction
Em

Figure 5. Northern blot analysis of *ermC* mRNA in the absence (-Em) and presence (+Em) of erythromycin. The numbers above the blot represent the time, in minutes, that samples were taken after the addition of rifampicin.


-Em
+Em
0 5 10 15
0 15 30 45 60 75 90 105 120
ermC wt

Chapter Two

Materials and Methods

Materials and Methods

2.1 Bacterial Strains

The strains of *B. subtilis* used as hosts for this study were BG1, which is *trpC2 thr-5*, and BG2, which is *hisA1 leu metB5*. The plasmids that they host are shown in Table II (Appendix A). Phage M13 was propogated in *E. coli* strain JM109, which is F' *traD36 lac*^{q} Δ(*lacZ*)M15*proAB/ recA1 endA1 gyrA96* (NA1^r) *thi hsdR17* (rk-mk+) *supE44 relA1* Δ(*lac-proAB*) (Yanisch-Perron *et al.*,1985).

2.2 Standard Procedures

The preparation of *B. subtilis* growth media and competent *B. subtilis* culture were as described (Dubnau and Davidoff-Abelson,1971). Transformation of competent *B. subtilis* was as described (Contente and Dubnau,1979). Selection for *B. subtilis* strains hosting the appropriate plasmids was done by plating transformed cells onto tryptose blood agar plates which contained either erythromycin (Em) or chloramphenicol (Cm) at a concentration of 0.02 and 5 μg/ml, respectively. Plasmid DNA was isolated from stationary phase cultures grown at 32° C overnight by the sodium dodecyl sulfate-NaCl method (Guerry *et al.*, 1973) and purified by CsCl-ethidium bromide density gradient centrifugation as described (Gryczan *et al.*,1978).

2.3 Small Scale Plasmid Preparation

Identification of *B. subtilis* strains hosting the appropriate plasmid DNA was performed as follows: 2 ml of minimal media (0.5% glucose, 0.1% casamino acids, 0.001% yeast extract, 0.001M $MgSO_4$, 0.5 mg/ml of His, Leu, Met, Thr, and Trp) containing 5 μg/ml of Cm or Em was grown overnight at 32° C. 1.5 ml of the overnight

culture was transferred to an Eppendorf tube and spun at 12,000g for 15 seconds. The supernatant was decanted and the cells were resuspended in 0.1 ml of a 0.5 mg/ml solution of lysozyme in 25% sucrose buffer (25% sucrose, 0.025 M tris pH7.5, 0.05M NaCl) by vortexing and incubated at 37° C for 15 minutes. Next, 24 µl of 5M NaCl: 6 µl of 0.5M EDTA pH 8.5: 135 µl of 2% SDS/0.7 M NaCl was added and the tubes were inverted gently to mix and incubated at 65° C for 10 minutes and then plunged into ice for 1.5 hours. The tubes were then spun for 15 minutes at 12,000g at 4° C and the supernatant was decanted to a fresh Eppendorf tube. 2 µl of 5 mg/ml Pancreatic RNase was added and incubated at 37° C for 30 minutes. 20 µl of predigested pronase (0.5 mg/ml in TES buffer) was added and the incubation was continued for another hour. 0.2 ml of a 1:1 solution of phenol-chloroform(phenol in TES pH7.5) was then added and the aqueous layer extracted after centrifugation. To precipitate the DNA, two volumes of ethanol was added and the solution was left on ice for 30 minutes, spun as above for 15 minutes, washed with 70% ethanol and lyophilized. The DNA pellet was then resuspended in 40 µl of sterile water and used for analysis.

2.4 Plasmids

Appendix A contains a list of the plasmids used for this thesis. Plasmid pBD246 encodes an *ermC-lacZ* translational fusion and its construction has been described (Gryczan *et al.*,1984). Plasmid pSD119 encodes the same *ermC-lacZ* fusion as pBD246, however, in this plasmid the SD2 of the *ermC* methylase has been mutated so that the sequence GAG of the ribosome binding site was changed to CCC and thus there is no

translation of the *ermC-lacZ* fusion (Bechhofer and Dubnau, 1987). Plasmid pSD121 contains a mutation of the -10 sequence of the *ermC* promoter which results in no transcription of the *ermC-lacZ* fusion. In this construct the -10 sequence was changed from 5'-TATAATTATA-3' to an *Eco*RI site, 5'-GAATTC-3'.

Fusions containing the 5' stem-loop structure of *ermC* and divergent downstream *B. subtilis* sequences were constructed as follows: To construct the plasmid expressing the *ermC-sacA* fusion, pSD215, plasmid pSD129 (Figure 6) was digested with *Bcl*I and *Eco*RI and this fragment was replaced with the *Eco*RI-*Bcl*I fragment of pSG8.1 (Fouet *et al.*, 1986) . Plasmid pSD215 encoded the first 360 nucleotides of *ermC* fused in frame with the terminal 900 nucleotides that encode the *B. subtilis sacA* and its transcriptional termination signal. Plasmid pSD222 (Figure 10) encodes an *ermC-sacB* fusion which was constructed by replacing the *Hpa*I - *Eco*RI fragment of pSD129 with the *Stu*I - *Eco*RI fragment of pLS302 (Steinmetz *et al.*,1985). This fusion encoded the first 730 nucleotides of the *ermC* mRNA fused in phase with the 1600 3'-proximal nucleotides of the *B.subtilis sacB* mRNA. The fusion plasmid for *ermC-epr* was pSD251 (Figure 10). To create this, plasmid pSD129 was digested with *Bcl*I and *Hin*dIII. This fragment was replaced with the *Bam*HI-*Hin*d III fragment of pNP2 (Sloma *et al.*,1988). The resulting fusion transcript contained the 360 5' proximal nucleotides of *ermC* fused in frame with the 1760 3'- proximal nucleotides of the *B. subtilis epr* gene. Plasmid pSD127 was a fusion containing the 360 5' proximal nucleotides of *ermC* transcript fused to the 40 3' proximal nucleotides of *penP* (Neugebauer et al.,1981) and was constructed previously by John La Polla (DiMari and Bechhofer, 1993).

Plasmid pSD212 contained a deletion of the transcriptional termination sequence of *ermC* which was removed by digesting pSD85 with *Hpa*I and *Bst*U1 and then ligating these blunt ends (Figure 6). This resulted in a deletion of the 180 3' proximal nucleotides of the *ermC* transcript including the sequence encoding the transcriptional terminator. The resulting plasmid is pSD212.

Sequences downstream of the 0.6 kb transcript of the *ermC-lacZ* fusion were removed by digesting plasmid pBD246 with *Cla*I. This digestion produced three fragments (Figure 6): The *Cla*I fragment flanking the *cat* (Chloramphenicol Acetyl Transferase) gene, the *Cla*I fragment containing the *ermC-lacZ* coding sequence to the *Cla*I site in *lacZ*, the fragment encoding the *lacZ* gene from the *Cla*I site to the end of *lacZ* and the downstream sequence to the *Cla*I site. After digestion with *Eco*RV, which cuts at a unique site in the *lacZ* coding sequence downstream of the *Cla*I site, the remaining *Cla*I fragments were religated. The ligation mixture was used to transform *B. subtilis* and colonies were selected for chloramphenicol resistance and the inability to cleave methyl-umbilliferyl β-galactoside (β -gal). This resulted in two plasmids since the remaining *Cla*I fragments could religate in two orientations. Plasmid pSD162 contained the two *Cla*I fragments in the same orientation as in pBD246 while plasmid pSD164 contained the two plasmids in the opposite orientation as pBD246 (Figure 13).

The sequence encoding the 3' terminus of the 0.6 kb transcript of the *ermC-lacZ* fusion is contained on a *Pvu*II-*Hpa*I fragment of pBD246. In order to construct the plasmid containing the *Pvu*II-*Hpa*I fragment of *lacZ* inserted into the *ermC* coding sequence, we took advantage of the fact that pBD246 contains the *Xba*I-*Bcl*I fragment of

pBD142 which encodes the 360 5' proximal nucleotides of *ermC*. This allowed us to replace the *Xba*I-*Hpa*I fragment of pBD144, a low copy derivative of pBD142, with the *Xba*I-*Hpa*I fragment of pBD246. The resulting plasmid contained the 360 5' proximal nucleotides of *ermC* transcript fused in phase, to a 412 nucleotide sequence of the *lacZ* coding sequence from the *Bam*HI to the *Hpa*I site followed by the 180 3' proximal nucleotides of the *ermC* transcript including the *ermC* transcriptional terminator. This plasmid was denoted as pSD191 (Figure 14).

Plasmid pSD229 contained a tandem repeat of the *Pvu*II-*Hpa*I sequence of *lacZ* inserted into the methylase coding sequence of *ermC*. To create this plasmid, pSD162 was digested with *Hpa*I and *Pvu*II. The 330 nucleotide *Pvu*II-*Hpa*I fragment was isolated by electroelution and ligated into plasmid pSD191 cleaved at a unique *Hpa*I site in the *ermC-lacZ* coding sequence (Figure 14).

The 16S-23S spacer region of *B. subtilis* ribosomal RNA operon *rrn*G was inserted into the *ermC* methylase coding sequence. Plasmid p14B8 (Bott and Hollis, 1982) was kindly provided by K. Bott. This plasmid contains a 500 nucleotide *Sma*I fragment which encodes the 16S-23S spacer region of the *rrnG* ribosomal operon. This 500 nucleotide fragment was ligated into the unique *Hpa*I site of plasmid pBD144 in two orientations. The resulting plasmids were pSD210 and pSD211, which contained this fragment in the same or opposite orientaion as the *rrnG* operon, respectively (Figure 17).

The 500 nucleotide *Sma*I fragment of p14B8 was cloned into the *Hinc*II site of M13mp7 RF which contains *Bam*HI sites on either side of the *Hinc*II site. RF DNA containing this construct was digested with *Bam*HI and this 520 nucleotide fragment was

isolated and ligated into the unique *BclI* site of plasmid pSD129, resulting in plasmids pSD216 and pSD217 which contained the *rrnG* DNA sequence in the same or opposite orientation as the ribosomal operon. The *Bam*HI fragment was also cloned into a *Bam*HI site of pGEM3Z(f+) and resulted in ligations of two orientations with plasmid pJFD2 containing the *rrnG* sequence in the same orientation as pSD216 and pJFD3 containing the insert in the same orientation as pSD217 relative to the T7 promoter.

Derivatives of pSD210 and pSD211 in which the ribosome binding site of the *ermC* methylase was inactivated by mutation were constructed by D.H. Bechhofer. The ribosome binding site for the *ermC* methylase contained the same mutation from a GAG to a CCC sequence in SD2 as pSD119. The pSD210 and pSD211 derivatives were denoted as pSD213 and pSD214, respectively.

To clone the *Bam*HI fragment of pJFD3 into the *BclI* site of the *lacZ* coding sequence in an *ermC-lacZ* translational fusion, pJFD3 was digested with *Bam*HI and the 520 nucleotide *Bam*HI fragment containing the *rrnG* coding sequence was isolated and inserted into the unique *BclI* site of pBD246. Plasmids containing this insert in both orientations were isolated and denoted as pSD227 and pSD228. pSD227 contained the *Bam*HI insertion so that it was transcribed in the same orientation as the *rrnG* operon and pSD228 contained the insert so that it was transcribed as the reverse complement of *rrnG*.

2.5 Bacteriophage SP82

Bacteriophage SP82 suspension was a gift from H.E. Hemphill, Syracuse University,

Syracuse,New York. Propogation of bacteriophage and isolation of SP82 DNA were performed as described (Hemphill,H.E., 1990).

Since no convenient restriction sites were available for cloning, polymerase chain reaction was employed to yield a fragment encoding a *B. subtilis* RNase III cleavage site flanked by *Bam*HI linkers. Oligonucleotides used as DNA primers were obtained from the DNA Core Facility of the Brookdale Center for Molecular Biology, Mount Sinai Medical Center, New York, New York. These primers contained sequences complimentary to nucleotides 242 to 257 and 517 to 501 of the SP82 left terminal repeat. Polymerase chain reactions were performed using Vent polymerase. The 370 base pair PCR product was isolated from a 5% polyacrylamide gel and reamplified using Taq polymerase and the above primers. The amplified PCR product was then isolated and digested with *Bam*HI and inserted into the unique *Bam*HI site of pGEM3Z(f+) to give pJFD4. The SP82 DNA sequence inserted into the *Bam*HI site was sequenced and was found to be only partially homologous with the original SP82 sequence (Figure 21). Since this fragment did encode the *B. subtilis* RNase III cleavage site, it was cloned into the *ermC* methylase coding sequence. Plasmid pJFD4 was digested with *Bam*HI and the isolated 370 nucleotide *Bam*HI fragment was cloned into the unique *Bcl*I site of pBD144. All of the clones obtained after transformation of BG2 cells with this DNA that contained the insert were identical. They all contained the insert so that it was transcribed the same as in SP82. One of these colonies was selected and its plasmid was denoted as pSD238. Successful cloning of the *Bam*HI fragment in the reverse orientation as SP82 was obtained using pBD142, which is the high copy version of pBD144. The resulting plasmid is pSD240.

2.6 Isolation and Analysis of RNA

The method employed for the isolation of RNA from *B. subtilis* strains and for Northern blot analysis was as described (Bechhofer and Dubnau, 1987). Cultures were grown with appropriate selection in minimal media overnight. These stationary cultures were then diluted 1:20 in minimal media without selection and grown as parallel cultures to the mid-logarithmic stage. Em (0.02 µg/ml) was then added to one of the parallel cultures and induction was allowed to proceed for 15 minutes. Rifampicin was added at a final concentration of 150 µg/ml and one ml samples were taken at various times and added to 0.5ml of frozen 50 mM $NaNH_3$. Total nucleic acid was isolated as described (Ulmanen *et al.*, 1985). For Northern blot analysis, the isolated nucleic acid was then treated with 20 units of RNase-free DNase and concentrations were assessed by spectrophotometric analysis at an OD 260/280.

For Northern blot analysis, 4 µg of RNA was loaded per lane. To check that equal amounts of RNA was loaded per lane and that the integrity of the RNA was good, gels were stained with ethidium bromide and photographed under ultraviolet light. The RNAs were then transferred to nitrocellulose membranes, hybridized overnight at 37° C, and then washed at 50° C. The 5' *ermC* probe used was constructed by inserting a DNA sequence from an *Mbo*I site upstream of *ermC* to an internal *Hae*III site (Figure 7) into M13mp19. The *ermC* 3' probe contained *ermC* sequences from the *Hpa*I site of *ermC* to a *Cla*I site downstream of the *ermC* transcriptional terminator cloned into M13mp19. Single-stranded DNA from the bacteriophage containing the *erm*C inserts was labeled by the reverse-priming method (Hu and Messing,1982). The labeled probe was separated

from free counts by passing it through a Sephadex G-50 spun column and added in excess during hybridization.

Northern blot analysis for the *ermC*-SP82 fusions were performed as above except that the hybridization and washing conditions were by the riboprobe method (Srivastava and Schonfeld,1991). Plasmid that were used for *in vitro* transcription of *ermC* riboprobes were constructed by K. K. Hue in Dr. Bechhofer's laboratory. Transcription from a T7 promoter yielded a 5' *ermC* probe complementary to the 5' proximal 360 nucleotides of *ermC*. The 3' *ermC* probe was also transcribed from a T7 promoter and was complementary to the 180 3' proximal nucleotides of *ermC*. The transcription products were treated with RQ1 RNase free-DNase and separated from unincorporated nucleotides by passage through a Sephadex G-50 column. Prehybridization was performed at 45° C as described for 5 hours, at which point probe was added and allowed to incubate overnight at 45° C. The blots were then washed at 65° C, air dried, and used for autoradiography.

2.7 Reverse Transcription Analysis

RNA was isolated from cells as described for Northern blot analysis with the omission of the DNase step. RNA was transcribed *in vitro* from the T7 promoter of pJFD4 linearized with *Xba*I, yielding an RNA identical to the SP82 Bs-RNase III sequence. An oligonucleotide complementary to nucleotides 246 to 260 of the SP82 PCR product was used as the primer for reverse transcriptase and was end-labeled using T4 DNA kinase and [γ-^{32}P]ATP. Reverse transcription was performed as described (Bechhofer and Zen, 1989) with the modification that various temperatures were used

during the elongation step of the primer extension reaction. 50 µg of cellular RNA or 0.1 µg of *in vitro* transcribed RNA was precipitated and resuspended in 11.5 µl of H_2O, 25 units of RNAsin and 2 µl of 10x Reverse Transcription Buffer (500 mM Tris-HCl pH 8.0, 80 mM $MgCl_2$, 300 mM KCl, 10 mM dithiothreitol). 4 ng of end-labeled primer was added and the samples were denatured for 10 minutes at 65° C. Samples were then shifted to 42° C for 5 minutes to anneal the primer to the template. Deoxynucleoside triphosphates were then added to a final concentration of 0.5 mM, 12 units of avian myoblastosis virus reverse transcriptase were added and the elongation reaction was carried out for 35 minutes at 42, 45 or 50° C. The reaction was terminated by adding EDTA to a final concentration of 50 mM. Nucleic acids were then isolated by extracting with a 1:1 phenol-chloroform solution and ethanol precipitating with a half-volume of 6M NH_4OAc and 2 volumes of ethanol. The DNA was pelleted, dried and resuspended in 4 µl of a 2:3 TE:Sequenase stop solution. Samples were then denatured at 80° C for 2 minutes and run on an 8% polyacrylamide sequencing gel along with a dideoxy sequencing reaction of plasmid DNA template using the same primer.

2.8 S1 Nuclease Analysis

Mapping of the 3' terminus of RNA decay intermediates was performed as described (Favaloro *et al.*,1980). The probes used to map the 3' terminus of the 0.6 kb transcript from pBD246 were labeled fragments from pSK10Δ6, which contained *lacZ* sequences from the *Bam*HI to the *Cla*I site or the *Bam*HI to the *Hpa*I site (Figure7). Plasmid pSK10Δ6 was digested with *Bam*HI and end-filled with [α-^{32}P]-dATP and cold deoxynucleoside triphosphates. The labeled DNA was then digested with either *Cla*I or

*Hpa*I and the 822 nucleotide *Bam*HI-*Cla*I fragment and 419 nucleotide *Bam*HI-*Hpa*I fragment were used as probes. Probes used for the 3' mapping of processed RNA from strains BE301 and BE303, which harbor plasmids pSD238 and pSD240, respectively, were made by digesting plasmid pJFD4 with *Bam*HI. The 370 base pair fragment encoding the SP82 PCR product was isolated and end-filled with [α-^{32}P] dATP and cold deoxynucleoside triphosphates. The probe for 3' end mapping of the BE301 processed RNA was made by digesting the 370 *Bam*HI fragment with *Nsi*I and isolating a 345 nucleotide *Bam*HI-*Nsi*I fragment. The probe for mapping strain BE303 RNA was made by digesting the 370 *Bam*HI fragment with *Taq*I and isolating a 340 nucleotide fragment. The specific activity of the probes ranged from 1.5×10^5 to 7.0×10^5 Cerenkov counts.

S1 analysis was performed as follows: 40,000 counts per minute of probe was added to 50 µg of total cellular RNA and the mixture was dried and resuspended in 10 µl of S1 hybridizaion buffer (80% (v/v) formamide, 0.4 M NaCl, 1 mM EDTA and 40 mM PIPES buffer pH 6.4. Hybridization was then carried out overnight at 42°C. 200 µl of S1 digestion buffer (250 mM NaCl, 40 mM NaOAc pH5.5, 1 mM $ZnCl_2$, and 20 µg/ml of denatured herring testes DNA were added and S1 digestion was performed using 400 units of S1 nuclease at 37°C for 30 minutes. The reaction was terminated by the addition of 4 µl of 10% sodium dodecyl sulfate, 20 µl of 0.2 M EDTA and 25 µl of 3 M NaOAc pH 8.0. Extraction was performed using phenol-chloroform (1:1) followed by ethanol precipitation. Dry samples were resuspended in 6 µl of TE and 4 µl of Sequenase stop solution, denatured at 80° C and run on a 6% polyacrylamide sequencing gel along with dideoxy sequencing reactions as DNA ladders.

2.9 β-Galactosidase Assays

β-Galactosidase assays were performed as described (Gryczan *et al.*,1984). Cells were grown overnight in the presence of 5 μg/ml Cm, diluted 1:40 in growth media and grown at 32° C under agitation to a Klett of 30 units in parallel cultures. Erythromycin was added to a final concentration of 0.02 μg/ml to one of the cultures and 45 seconds later rifampicin was added to a final concentration of 150 μg/ml. Duplicate one ml samples were taken at various times, spun down and resuspended in 1X Spizizen salt solution, spun again and resuspended in one ml of assay buffer (0.1 M $NaPO_4$, pH 7.0, 1.0 mM $MgSO_4$, and 0.1 M β-mercaptoehanol). 50 μl of toluene was added, the samples were vortexed and placed on ice. 0.2 ml of the sample was transferred to a tube containing 0.6 ml of assay buffer and preincubated at 30° C for 5 minutes. 0.2 ml of 4.0 mg/ml of ONPG was added and the reaction was allowed to continue until a yellow color appeared. The time of the reaction was recorded and the reaction was stopped by addition of 0.5 ml of 1.0 M Na_2CO_3. Samples were then centrifuged to remove cells and an absorbance at 420 nm was taken. β-galactosidase units were defined as:

$$\frac{A_{420} \times 1000 \times \text{Time of Reaction (seconds)}}{\text{Klett}}$$

2.10 Densitometry

Densitometry was performed by scanning Northern blots with a Xerox Dataplus scanner. The scanned image was converted into a bit mapped image by the software package Mac Draw 2.0. The relative area for the RNA bands was determined by using the program Image 1.43.

2.11 DNA Sequencing

DNA sequencing was performed using the dideoxynucleotide chain termination method described in the Sequenase version 2.0 Sequencing kit purchased from United States Biochemical Corp. Sequencing of DNA fragments cloned into bacteriophage M13 was performed using single-stranded DNA isolated from cultures of JM109 infected with the appropriate phage supernatant. 1.5 ml of the inoculated culture was incubated at 37° C with shaking for 5 hours. The cultures were transferred to eppendorf tubes and spun for 5 minutes by centrifugation to remove cells. The supernatant was transferred to a fresh eppendorf tube and recentrifuged as above. The supernatant was then added to 0.2 ml of polyethylene glycol/NaCl and incubated at room temperature for 15 minutes. The solution was centrifuged as above and the supernatant discarded. The samples were spun for an additional 2 minutes and the remaining supernatant was removed with a drawn pasteur pipet. The pellet was resuspended in 0.1 ml of TE buffer and 50µl of phenol/TE was added. The samples were vortexed for 20 seconds, let stand at room temperature, and vortexed again for 20 seconds. The samples were centrifuged for 3 minutes and the aqueous layer was transferred to a fresh eppendorf tube and ethanol precipitated. The samples were washed with 70% ethanol and dried under vacuum. Samples were

resuspended in 10 µl of TE and 1-2 µg was used for sequencing. Sequencing was performed as described using primers specific for the sequence flanking the M13 polycloning region. Sequencing of DNA fragments inserted into the polycloning region of pGEM3Z(f+) was performed on CsCl purified plasmid DNA. The protocol was essentially the same as that of the Sequenase kit with some modifications (Bechhofer,1991). 1 µg of DNA was added to 2 µl of 5X Sequenase buffer, 20 pmoles of primer, and 0.4 µl of 10% nonidet P-40. The volume was brought to 10 µl, boiled for 3 minutes and snap-cooled in dry ice. The other components for sequencing were added on ice and the reaction was performed according to the Sequenase protocol.

2.12 Southern Blotting

Southern blotting was performed as follows: chromosomal DNA was digested with the appropriate restriction endonucleases (*Bcl*I and *Bam*HI for DNA isolated from srain BE174). The DNA treated with pancreatic RNase and was subject to electrophoresis on a 0.8 % agarose gel. The gel was stained with ethidium bromide and the integrity of the DNA was examined by visualization under an ultraviolet lamp. The gel was soaked in 0.25 M HCl for 12 minutes and then washed twice with water. The gel was then soaked in 0.5 M NaOH for 60 minutes and washed twice with water. The gel was then soaked in a solution of 0.5 M Tris pH 9.0 and 1.5 M NaCl for 60 minutes. The gel was then placed on a piece of nitrocellulose that had been soaked in 6X SSC and the DNA was transferred by gravity overnight using 20X SSC as the transfer media. The nitrocellulose membrane was then soaked in 6X SSC for 10 minutes and dried for 2

hours at 80° C in a vacuum oven. The blot was prehybridization in a solution containing 50% formamide, 4.8% dextran sulfate, 2.4X SSC, 0.1% SDS, 1X Denhardts and 1.5 mg/ml of salmon sperm DNA for 8 hours at 37° C. Hybridization was performed by incubation at 37° C overnight in a solution of 50% formamide, 10% dextran sulfate, 2X SSC, 1% SDS, 5 mM EDTA pH8, and 1 mg/ml salmon sperm DNA. The blot was then soaked in a solution of 2X SSC and 0.5% SDS at 24° C for 5 minutes, transferred to a solution of 2X SSC and 0.1% SDS for 15 minutes at 24° C, and then washed for 2 hours at 55° C in a solution of 0.1X SSC and 0.5% SDS. The blot was then air dried and placed in an x-ray cassette with a screen and Kodak XAR film for 2 days at -70° C.

The probe for the ermC gene contained in BE274 was prepared as follows: 50 µg of A3 Turn Around DNA (a single stranded M13 template containing a sequence complementary to an internal 470 nucleotides of *ermC* mRNA) was mixed with 10 µl of 10X Klenow buffer and 10 µg of HP primer in a total volume of 50 µl. The DNA was denatured at 90° C for 3 minutes, cooled to 65° C for 10 minutes and annealed at 24° C for 15 minutes and stored at -20° C. 14.2 µl of the primer annealed template was mixed with 2 µl of 250 µM dCGTTP and 3 µl of a-^{32}P-dATP and 1 unit of Klenow Enzyme. The DNA polymerase reaction was allowed to continue for 1 hour at 24° C. The probe was separated from free nucleotides as described for Northern blotting.

Chapter Three

Results

Results

3.1 Transcriptional Fusions

In the presence of Em-bound ribosomes, the 5' proximal sequence of the *ermC'* gene acts *in cis* to confer stability to the entire *ermC'* mRNA. We wished to determine whether this induction of stability was specific for the *ermC'* sequence or if the 5' proximal region could act *in cis* to stabilize any downstream sequence. To test this, various fusions described below were constructed containing the 5' stabilizing sequence of the *ermC'* gene and diverse downstream sequences. Northern blot analysis was performed on RNA isolated from *B. subtilis* strains harboring plasmids containing these transcriptional fusions, in the presence and absence of Em-bound ribosomes. The ability of the 5' proximal region of *ermC'* to induce stability *in cis* to different downstream sequences in the presence of Em-bound ribosomes was demonstrated.

3.1.1 Stabilization of an *ermC-lacZ* Transcriptional Fusion

Plasmid pBD246 (Figure 6) contained the *ermC'* gene of plasmid pBD142 fused in frame to the *E. coli lacZ* gene, which encodes β-galactosidase (Gryczan *et al*.,1984). This fusion contained the 5' proximal nucleotides of the *ermC'* gene up to the *BclI* site fused to the *lacZ* gene at the *Bam*HI site. A schematic of this fusion is shown in Figure 7. RNAs transcribed from this fusion contained the first 360 nucleotides of *ermC'* which included the entire *ermC'* leader peptide coding sequence as well as the *ermC'* methylase coding sequence up to codon 73, fused in frame with the *lacZ* coding sequence starting from codon eight. Previous experiments have demonstrated that β-galactosidase activity from

this fusion was inducible by stalling of Em-bound ribosomes in the leader peptide coding region of *ermC'* (Gryczan *et al* , 1984*)*. Simultaneous with this increase in translation, an increase in *ermC'* mRNA stability was observed (Bechhofer and Dubnau, 1987) Analysis of transcripts from pBD246 carried in strain BE8 demonstrated that three major RNA species were detectable using an *ermC'* 5' probe and these RNAs were approximately 0 6, 4 and 6 kb in size (Figure 8A) The 0 6 and the 4 kb RNAs were stabilized in the presence of Em-bound ribosomes while the 6 kb RNA showed no induced stability

These results indicated that the 5' regulatory region of the *ermC'* gene can act *in cis* to confer stability to the downstream *lacZ* sequence During induction by Em-bound ribosome stalling in the 5' leader region, an increase in translation occurs due to an increased accessibility of the SD2 site It could be argued that the increase observed in RNA stability is due to protection of the transcripts by an increased density of ribosomes moving along the RNAs If this were true then the stability of the transcripts would not be due to stalled ribosomes in the 5' leader region, but to the inaccessibility of the transcript to RNases while high-level induced translation is occurring To address this, a mutation of pBD246 was made in which the SD2 sequence was altered from AGAGGG to ACCCGG (Figure 3) Previous experiments demonstrated that this mutation renders the SD2 sequence nonfunctional (Bechhofer and Dubnau, 1987) The strain harboring the *ermC'-lacZ* fusion with this mutation was BE152 To insure that translation from the SD2 sequence as well as from any possible internal *lacZ* sequence was not occurring in BE152, β-galactosidase assays were performed using strain BE8 as a positive control and strains BG2 and BE154 as negative controls Strain BG2 contained no *lacZ* gene and was a

control for endogenous β-galactosidase activity in *B. subtilis* (Errington and Vogt, 1990) BE154 contained plasmid pSD121, which was identical to pBD246 with the exception that the *ermC'* promoter was inactivated by mutating the -10 sequence from TATAATTATA to an *Eco*RI site (GAATTC). The results of the β-galactosidase assays are shown in Figure 9. It was clearly demonstrated that strain BE8 contained β-galactosidase activity that was inducible in the presence of Em-bound ribosomes. Strains BE154 and BE152 expressed no β-galactosidase activity detectable above background. These results demonstrate that the *ermC'-lacZ* coding sequence of BE152 was not being translated.

Once it was established that strain BE152 did indeed contain a nonfunctional SD2 sequence, Northern blot analysis was performed in the presence and absence of Em on strains BE8 and BE152. Figure 8B shows the results of this experiment. Analysis of RNA isolated from both strains demonstrated that three major species were detected using an *ermC'* 5' probe. The 6 kb RNA decay was unaffected by the presence of Em while the 0.6 and 4 kb RNAs were inducibly stable in the presence of Em-bound ribosomes and were detectable even at 100 minutes after rifampicin addition. The induction of RNA stability by Em in strain BE152 demonstrated that the induced stability was not dependent on translation of the *ermC'-lacZ* coding sequence.

The presence of intermediate-sized minor bands detectable by Northern analysis of BE8 and BE152 RNA may represent RNA decay intermediates or transcripts from a weak promoter proximal to that of *ermC'*. To differentiate between these two possibilities, Northern blot analysis of RNA isolated from strain BE154, which contained the -10

mutation was done with BE152 as a positive control for hybridization The intermediate bands detected upon Northern analysis of BE8 and BE152 RNA located between the 4 and 0 6 kb bands were not detectable, demonstrating that they did originate from the *ermC'* promoter and are probably decay intermediates (data not shown)

3.1.2 Stabilization of Downstream *B. subtilis* Sequences

The long half-lives of the inducibly stable *ermC'-lacZ* RNAs was rather impressive The observation that these transcripts remained stable during Em induction independent of translation indicated that the stability was not due to ribosomal protection of the body of the message How then does ribosomal stalling in the 5' leader region of in the *ermC'-lacZ* transcripts protect the entire RNA? Do these results demonstrate that the initiation of decay must occur at the 5' proximal region of an mRNA in *B. subtilis* or was there something special about the *lacZ* sequence? Since the *lacZ* sequence was from *E. coli*, it is possible that the transcripts from the *ermC'-lacZ* fusion were inducibly stable due to an absence of recognition signals for the initiation of decay in *B. subtilis*. To address this, fusions were constructed which contained the *ermC'* 5' regulatory region fused to the 3' proximal region of various *Bacillus* genes The schematic representation of these fusions is shown in Figure 10

The mRNA transcribed from the *penP* gene of *Bacillus licheniformis* has a half-life of about two minutes A previous report had suggested that the short half-life of the *penP* transcript was dependent on its 3' proximal sequence (Wong and Chang, 1986) The 40 3' proximal base pairs of *penP* were fused to the 360 5' proximal base pairs of

ermC' at their *BclI* sites to determine whether the 5' regulatory region of *ermC'* could impart stability to a RNA terminating with a sequence that was thought to be a decay determinant Northern blot analysis of RNA isolated in the presence or absence of Em from strain BE168, which hosts the *ermC'-penP* fusion, is shown in Figure 11A The transcript of this fusion was 400 nucleotides in length and was inducibly stable in the presence of Em-bound ribosomes Densitometric analysis was performed from Northern blots of this RNA and the results are given in Table 1 In the absence of Em the half-life of the *ermC'-penP* transcript was determined to be approximately 4 5 minutes In the presence of Em the half-life of this transcript was increased to approximately 17 minutes Therefore, the presence of a 3' terminal sequence, which confers a two minute half-life to its own mRNA does not interfere with the induced stability conferred to the transcript by the 5' regulatory region of *ermC'*

The stability of the *ermC'-penP* fusion transcript may have been due to the length of the transcript Since the fusion only contained 40 nucleotides of *penP* sequence, it was possible that the *ermC'-penP* fusion contained no RNase cleavage sites in the *penP* region To address this and to examine the generality of this finding, fusions were constructed containing the 5' regulatory region of *ermC'* fused, in frame, to lengthy 3' downstream sequences of several *Bacillus* genes An *ermC'-sacA* fusion was constructed which contained the first 360 nucleotides of the *ermC'* gene fused at the *BclI* site to the 900 base pairs at the 3' end of the *sacA* (sucrase) gene of *B. subtilis* (Fouet *et al.*,1986) The last 1,760 nucleotides of the extracellular protease (*epr*) gene of *B. subtilis* (Sloma *et al.*,1988) was also fused to the 360 5' proximal nucleotides of *ermC'* mRNA Plasmid

pSD222 contained an *ermC'-sacB* fusion whose transcript contained the first 740 nucleotides of *ermC'* and the 1,600 downstream nucleotides of the *B. subtilis sacB* (levansucrase) mRNA. Finally, the transcriptional terminator of *ermC'* was removed by digesting plasmid pSD129 with *Hpa*I and *Bst*UI to examine the stability of an *ermC'* mRNA with a different 3' end. Northern blot analysis was performed on RNA isolated from strains harboring these fusions at various times after the addition of rifampicin in the presence or absence of Em. The results of this analysis are shown in Figure 11. A single transcript was detected for the *ermC'-sacA*, *ermC'-epr* and *ermC'-sacB* fusions and these mRNAs were all inducibly stable in the presence of Em-bound ribosomes. The half-lives of these mRNAs was determined by densitometry and are listed in Table 1. The half-lives of the mRNAs for *sacA*, *epr*, *penP* and *sacB* were found not to be affected by Em.

Two RNAs were detected by Northern analysis of RNA isolated from the strain hosting the *ermC'* terminator deletion mutant. The larger one was estimated to be about 3 kb in length and the smaller one about 1 kb. The 3 kb RNA appeared to be in greater abundance than the 1 kb RNA and both of these were inducibly stable by the addition of Em. The 3 kb RNA was determined to have a half-life of 3.8 minutes in the absence of Em and 10 minutes in the presence of Em. The half-life of the 1 kb RNA was 7.5 minutes in the absence of Em and was not determined in the presence of Em since it was found to increase in concentration during the time tested. This implies that the 1 kb RNA was formed by the processing of the 3 kb RNA.

We conclude from the above results that the 5' regulatory region of the *ermC'* gene acts as a general "5' stabilizer" and that this effect is independent of downstream

sequences The stabilizing effect of the *ermC* 5' leader region is imparted by its ability to act as a stalling site for Em-bound ribosomes These data strongly indicate that the initiation of mRNA decay in *B. subtilis* occurs at the 5' proximal region

Figure 6 Schematic of plasmids pBD246, pBD142, pSD85 and pSD129 The antibiotic resistance genes carried by these plasmids is shown as well as the direction of their transcription Restriction endonucleolytic sites that are not represented more clearly in following figures are shown pSD129 is the low copy version of pSD85, otherwise these plasmids are identical The polylinker region of pSD85 and pSD129 resides in the small *Cla*I fragment

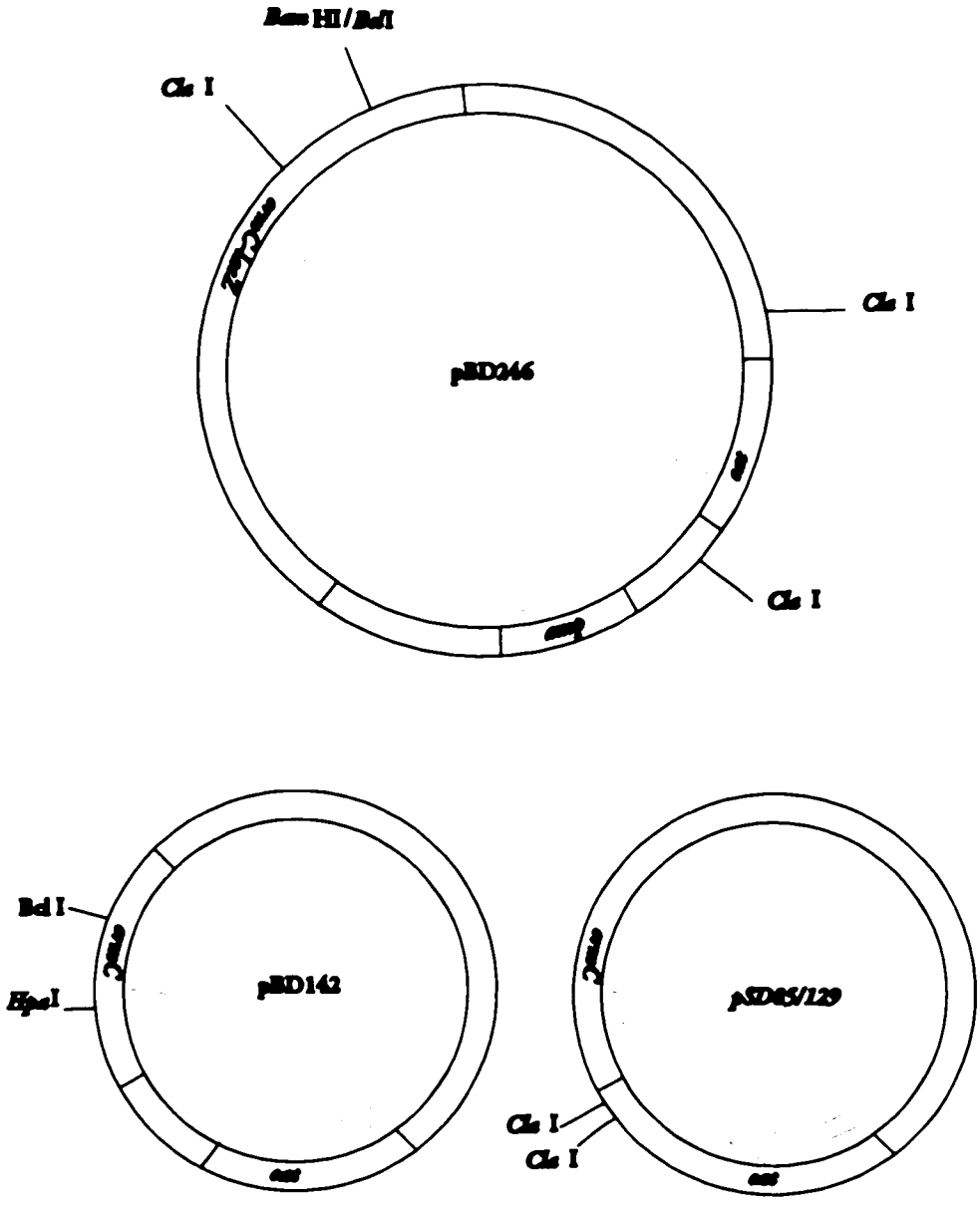

BamHI/BclI
ClaI
ClaI
ClaI
pBD246
BclI
HpaI
pBD142
pBD85/129
ClaI
ClaI

Figure 7 Schematic of the *ermC'* and *ermC'-lacZ* genes The open reading frames for the leader peptide coding sequence (lpcs) and the *ermC'* methylase are indicated by the filled bars The ribosome binding sites (SD) for these peptides are indicated as well as the *ermC'* transcriptional promoter (P) and terminator (T) The *ermC'-lacZ* fusion of pBD246 is also shown The coding sequence for the *lacZ* gene is represented by the stippled bar Restriction sites are shown for *Bam*HI (Bam), *Bcl*I (Bcl), *Cla*I (Cla), *Hpa*I (Hpa), and *Pvu*II (Pvu) The two inducibly stable RNAs from the *ermC'-lacZ* fusion are shown (0 6 kb and 4 kb)

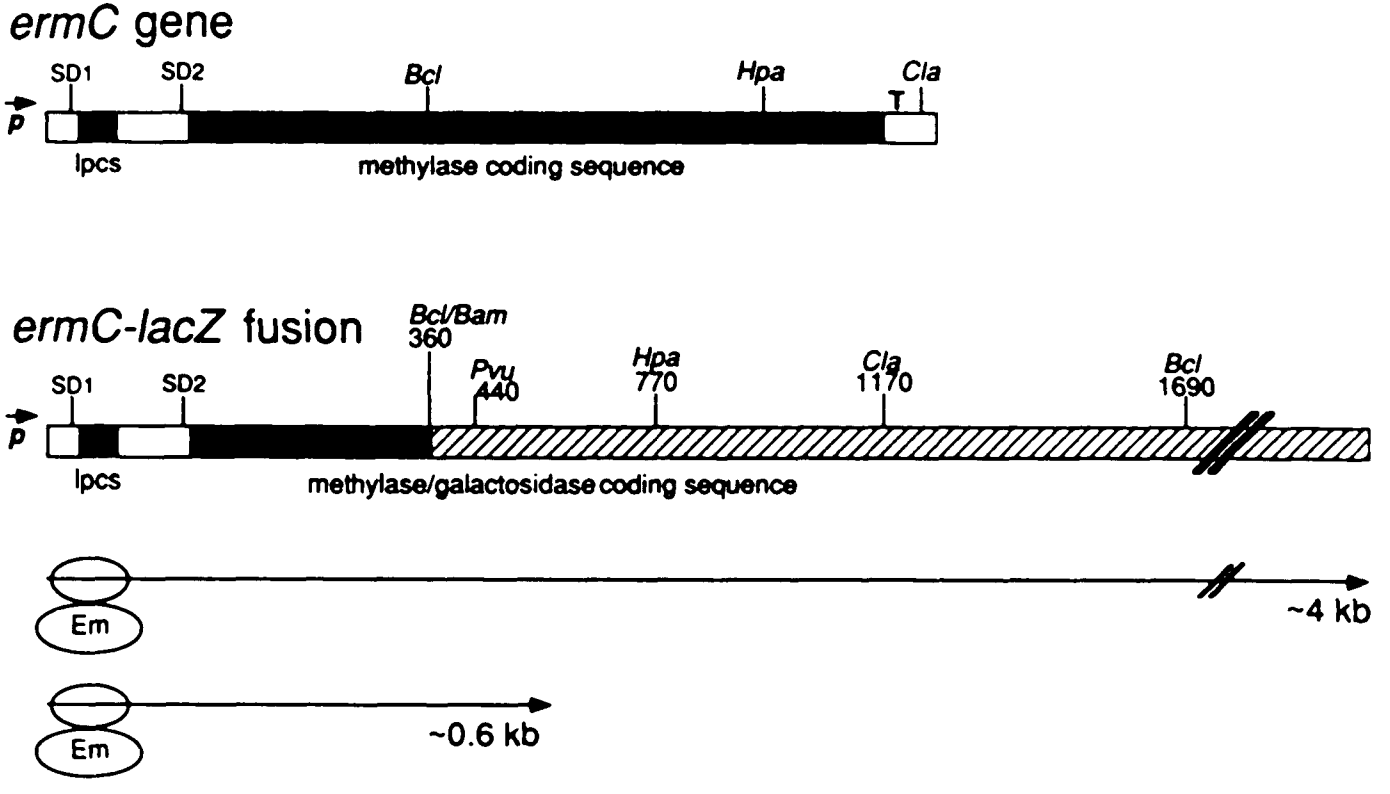

ermC gene
SD1
SD2
BcI
Hpa
Cla
P
lpcs
methylase coding sequence
ermC-lacZ fusion
BcI/Bam
360
Pvu
440
Hpa
770
Cla
1170
BcI
1690
SD1
SD2
P
lpcs
methylase/galactosidase coding sequence
Em
~4 kb
Em
~0.6 kb

Figure 8 (A) Northern blot analysis of RNA isolated from strain BE8, which harbored plasmid pBD246 Samples were taken at various times after the addition of rifampicin in the presence and absence of Em The time (in minutes), that each sample was taken after rifampicin addition is shown Approximate sizes of the two prominent inducibly stable RNAs are shown (B) Northern blot analysis of RNA isolated from strain BE152, which harbored plasmid pSD119, an SD2 mutant of pBD246 The blot is labeled in the same manner as in Figure 8A

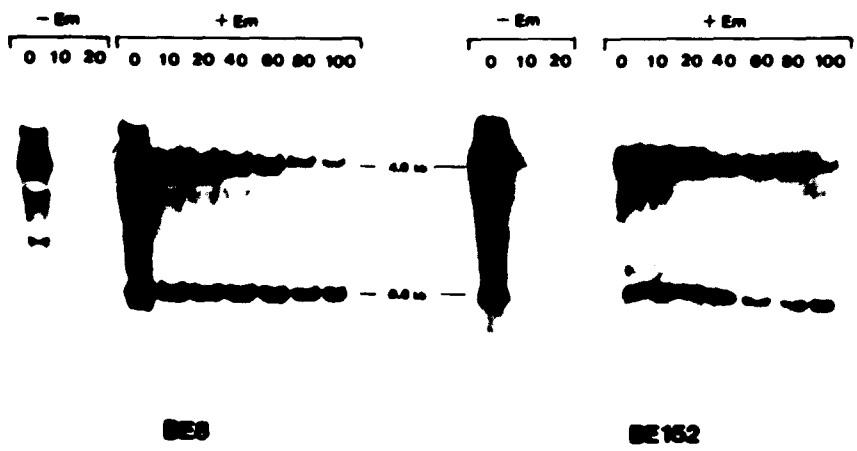

- Em
+ Em
0 10 20
0 10 20 40 60 80 100
- Em
+ Em
0 10 20
0 10 20 40 60 80 100

Figure 9 Induction of β-galactosidase activity in the *ermC'-lacZ* fusion strains by addition of 0.02 μg/ml of Em. Samples were taken at the indicated times after the addition of Em and assayed for β-galactosidase activity. Strain BE8 contained the *lacZ* fusion to the wild-type *ermC'* leader region. BE152 contained the *lacZ* fusion to the *ermC'* leader region containing an SD2 mutant. BE154 contained the *ermC'-lacZ* fusion with a mutated promoter sequence. BG1 contained no plasmid.

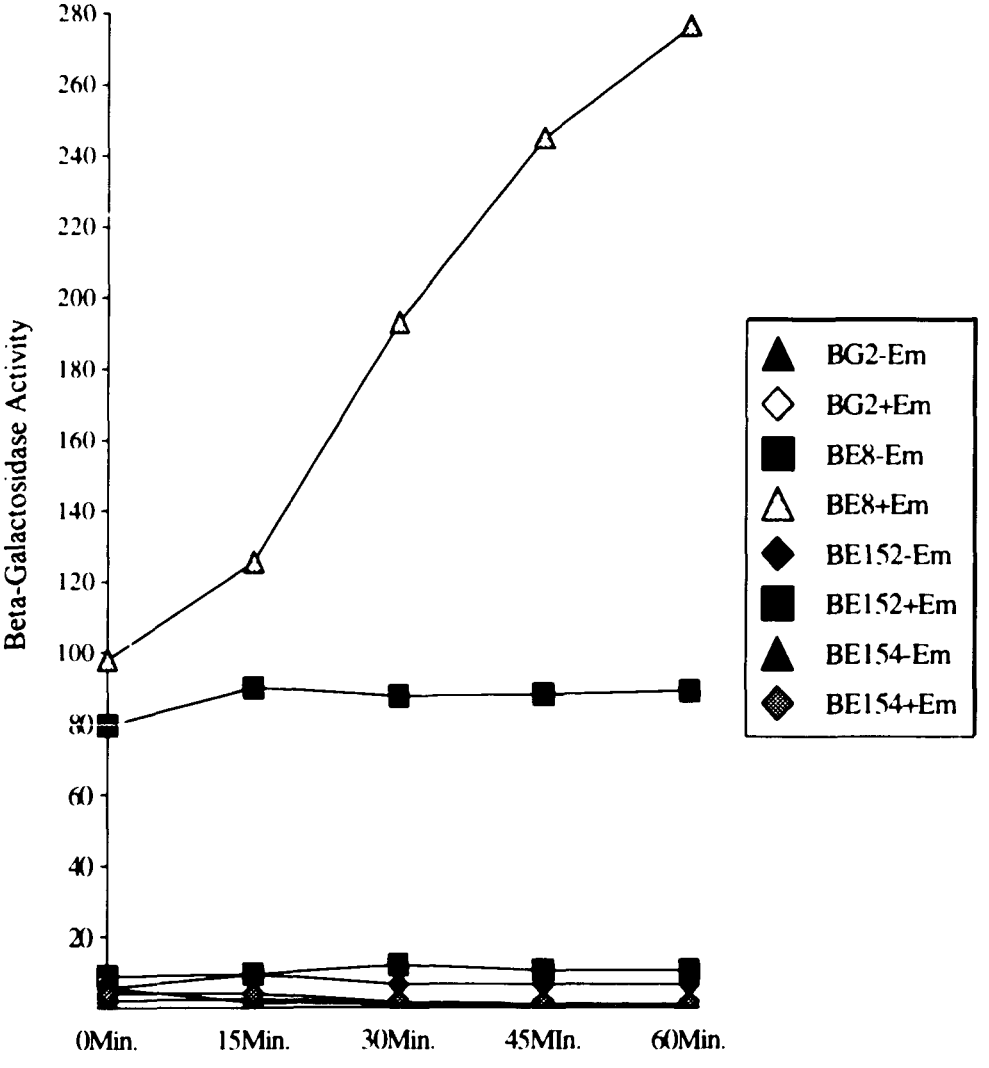

Beta-Galactosidase Activity
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0Min.
15Min.
30Min.
45MIn.
60Min.
BG2-Em
BG2+Em
BE8-Em
BE8+Em
BE152-Em
BE152+Em
BE154-Em
BE154+Em

Figure 10. Schematic of the *ermC'* transcriptional fusions. The transcriptional promoter for each fusion is shown (P) as well as the transcriptional terminator (t). The direction of transcription is indicated by the arrows. The site of fusion with the *ermC'* gene is indicated by the appropriate restriction site. The open bars containing the arrows represent *ermC'* sequence and the non-*ermC'* sequences are represented by filled bars. The name of the fusion and the plasmid on which it was contained, is shown.

ermC-penP (pSD127)

ermC-epr (pSD215)

ermC-sacA (pSD251)

ermC-sacB (pSD222)

ermC Δ terminator (pSD212)

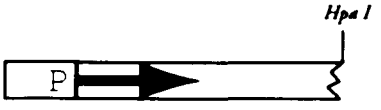


Figure 11 Northern blot analysis of the *ermC'* fusions Parallel cultures were grown in the absence (-) and presence (+) of Em Samples were taken after the addition of rifampicin at various times (in minutes), as indicated above each lane The estimated size (in nucleotides) of the RNAs from these fusions is indicated to the left of each blot The migration of the *ermC'* wild-type mRNA is indicated (910) The estimated sizes for the *ermC'* transcriptional terminator deletion (E) were based on control RNAs run in parallel lanes (not shown) The fusions were (A) *ermC'-penP*, (B) *ermC'-sacA*, (C) *ermC'-epr*, (D) *ermC'-sacB*, (E) *ermC'* transcriptional terminator deletion

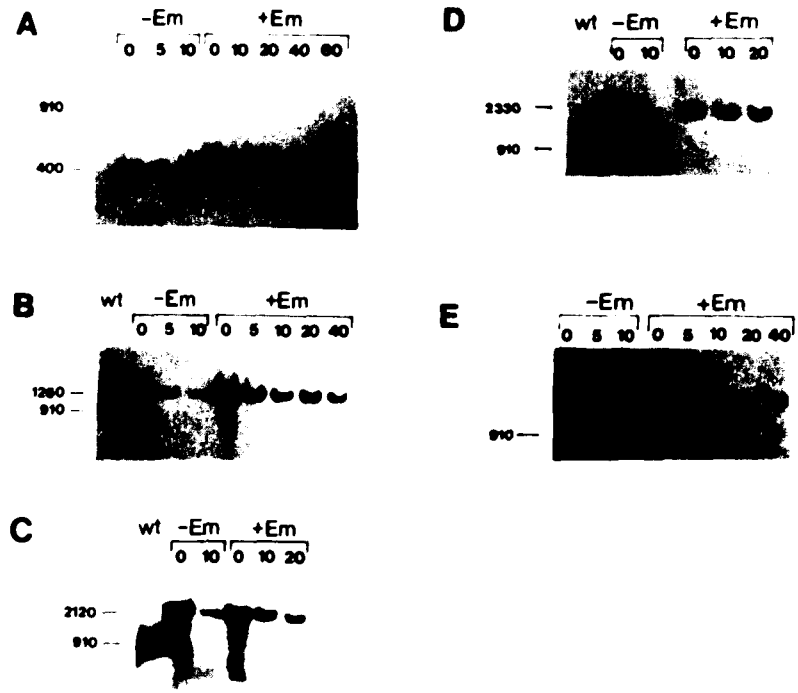

A
-Em
+Em
0 5 10 0 10 20 40 60
910
400
B
wt
-Em
+Em
0 5 10 0 5 10 20 40
1260
910
C
wt
-Em
+Em
0 10 0 10 20
2120
910
D
wt
-Em
+Em
0 10 0 10 20
2330
910
E
-Em
+Em
0 5 10 0 5 10 20 40
910

Table I Table of the half-lives of the *ermC'* fusions in the absence (-) and presence (+) of Em

Table I

Plasmid	Fusion	Half-life -Em	Half-life +Em
pBD142	*ermC* (wt)	2 0 min	25 min
pSD127	*ermC'-penP*	4 5 min	17 min
pSD215	*ermC'-epr*	7 0 min	17 min
pSD251	*ermC'-sacA*	3 0 min	17 min
pSD222	*ermC'-sacB*	2 5 min	>20 min
pSD212	*ermC'* Δ terminator Top	3 8 min	10 min
	ermC' Δ terminator Bottom	7 5 min	N D

3.2 Characterization of the 0.6 Kb RNA of *ermC-lacZ*

The 0 6 kb RNA of the *ermC-lacZ* fusion could either be a product of transcriptional termination or RNA processing The possibility of this transcript being a product of RNA processing made it of interest to us since the processed product remained stable in the presence of Em This would indicate that the process of mRNA decay in *B. subtilis* is not an all or nothing event The 0 6 kb RNA may contain some feature which blocks the decay process For this reason it was desirable to map the 3' terminus of this transcript

3.2.1 S1 Mapping of the 3' Terminus of the 0.6 Kb RNA

In order to define the 3' terminus of the 0 6 Kb transcript, S1 analysis was employed Two probes were used for the S1 analysis an 822 bp *Bam*HI-*Cla*I fragment and a 419 bp *Bam*HI -*Hpa*I fragment of the *lacZ* coding sequence that were labeled by end-filling (Figure 7) The construction of these probes and the method of analysis were as described (Materials and Methods)

The results of the S1 analysis are seen in Figure 12 RNA was isolated from strain BE8, which carried the *ermC-lacZ* fusion plasmid, in the presence of Em at the time of rifampicin addition (lane 2) and 40 minutes after the addition of rifampicin (lanes 3 and 5) RNA was also isolated from strain BG1 (no plasmid) as a control Analysis of the RNA isolated from BE8 at the time of rifampicin addition (lane 2) revealed a smear, which may represent the intermediates of degradation of the longer *ermC-lacZ* RNAs A smear was also detected by Northern blot analysis of BE8 RNA and was found to decrease in

intensity over time (Figure 8A) Analysis of the RNA isolated 40 minutes after the addition of rifampicin (Figure 12, lanes 3 and 5) demonstrated a decrease in background (the smear) as was anticipated from the Northern blot analysis and also revealed a cluster of prominent bands that were also visible at the zero time point This cluster of bands represents a series of 3' termini that map from positions 564 to 575 from the start of transcription of the *erm(*'*-lacZ* fusion A series of faint bands was detectable above this cluster of discrete bands and these may represent the faint bands detectable upon Northern blot analysis of BE8 RNA Although this analysis reveals that the 0 6 kb RNA was really a set of RNAs with slightly different 3' ends, this set of bands will be referred to as the 0 6 kb RNA for convenience

3.2.2 Formation of the 0.6 Kb RNA 3' End

The above results suggested that the 3' termini of the 0 6 kb transcript was a product of RNA processing Detection of a smear at the zero time point with a decrease in intensity over time to yield a cluster of smaller bands is the result one would expect if the 0 6 kb RNA was produced by a processive degradation of longer transcripts in the 3'-to-5' direction

Although formation of the 0 6 kb RNA by exoribonucleolytic processing is consistent with the data, one could not rule out the possibility of the 3' ends of this "RNA" being formed by transcriptional termination Determining whether processing or termination leads to the formation of the 3' end of an RNA *in vivo* is difficult to do Here I present results that strongly indicate that the 0 6 kb RNA was a product of

post-transcriptional processing

3.2.3 Removal of Sequences Downstream of the 0.6 kb 3' Terminus

If the 0 6 kb RNA was a product of transcriptional termination, then removal of sequences downstream of the 3' terminus of the 0 6 kb RNA would not be expected to affect the formation of this transcript However, if this transcript did arise from post-transcriptional RNA processing, then an alteration in sequences downstream of the 3' terminus could have an effect of the formation of this transcript A deletion was made of the sequences downstream of the *Cla*I site of the *lacZ* coding sequence (Figure 6) This deletion would not be expected to affect the formation of the 0 6 kb RNA if this transcript did indeed arise from a transcriptional terminator since the deletion was 600 nucleotides from the mapped 3' terminus of the 0 6 kb RNA

Plasmid pBD246 was digested with *Cla*I and religated Strains were isolated in which the fragment containing the sequence downstream of the *lacZ Cla*I site was deleted These strains contained the *Cla*I fragment flanking the *cat* gene in either the same or the opposite orientation as pBD246 (Figure 13) Strain BE223 contained the *cat* gene in the same orientation as pBD246 Northern blot analysis of RNA isolated from this strain revealed that three bands were detected (data not shown) The smallest of these bands corresponded with the 0 6 kb RNA seen in RNA isolated from strain BE8, which harbored plasmid pBD246 However, Northern blot analysis of RNA isolated from strain BE224, which contained the *cat* gene in the opposite orientation as pBD246, revealed that no band corresponding to the 0 6 kb RNA was detectable (data not shown) These results indicated

that the 0 6 kb RNA was a product of RNA processing and not transcriptional termination If the 0 6 kb RNA was a product of transcriptional termination it should have been detected in both BE223 and BE224 since both strains contained plasmids identical up to 600 bp downstream of the 0 6 kb RNA 's 3' terminus Differences in sequence this far away should not affect a transcriptional terminator The presence of the 0 6 kb RNA in BE223 could be due to initiation of processing at sequences encoded downstream of the *ClaI*, and this initiation site may not exist in the fusion RNA from BE224

3.2.4 Insertion of the 3' Terminal Sequence of the 0.6 kb RNA into *ermC*

Further experiments were done to distinguish whether the 0 6 kb RNA was a product of transcriptional termination or RNA processing It was decided that insertion of the sequence encoding the 3' terminus of the 0 6 kb RNA into a defined transcriptional unit might be of some assistance in elucidating this problem If the 0 6 kb RNA was produced by transcriptional termination, then insertion of the sequence encoding the terminator upstream of the normal terminator for a gene should reduce the amount of RNA encoding the 3' terminus of that gene However, if the 3' terminus of the 0 6 kb RNA was formed by a processing stop site, then insertion of this sequence upstream of a stable RNA sequence should lead to a decrease in the formation of the 3' terminus of the 0 6 kb RNA The 3' terminal sequence was inserted into the *ermC* coding sequence by replacing the 370 base pair *BclI-HpaI* fragment of *ermC* with the 412 base pair *BamHI-HpaI* fragment of *lacZ* (Figure 14) The resulting plasmid was pSD191 and was harbored in strain BE253 Northern blot analysis was performed on RNA isolated from strain BE253

The results of this analysis can be seen in Figure 15A and a schematic of the detectable bands can be seen in Figure 14 Northern blot analysis demonstrated that the major transcript detected with a 5' *ermC'* probe was an approximately 940 nucleotide transcript, corresponding to the expected full length transcript A second minor transcript was also detectable which was approximately 600 nucleotides in length and this corresponded to the 0 6 kb transcript detected from the *ermC'-lacZ* fusion of pBD246 Both of these transcripts were inducibly stable in the presence of Em-bound ribosomes, however, the induced stability of the 0 6 kb RNA was rather poor compared to the 940 nucleotide RNA The results of this experiment demonstrated that the *Bam*HI-*Hpa*I fragment of *lacZ* is sufficient for the formation of the 0 6 kb RNA when inserted into the *ermC'* transcript but did not allow us to distinguish between transcriptional termination or RNA processing as being the cause for the formation of this transcript

To distinguish between these two possibilities a tandem duplication of the *lacZ* *Pvu*II-*Hpa*I fragment was inserted into the *ermC'* coding sequence Plasmid pSD191 was linearized at its unique *Hpa*I site and a 330 bp *Pvu*II- *Hpa*I fragment isolated from pSD191 (Figure 14) was inserted into this site The resulting plasmid was pSD229 and a schematic of this construct is given in Figure 14 The strain harboring this plasmid was BE292

Northern blot analysis was performed on RNA isolated from strain BE292 and the result of this analysis is shown in Figure 15B A schematic of these results is shown in Figure 14 Three RNAs were detected, which were inducibly stable in the presence of Em the full-length 1,650 nucleotide transcript with the 3' terminus of *ermC'*, an 890 nucleotide

RNA terminating at the downstream inserted sequence, and a 560 nucleotide RNA terminating in the upstream inserted sequence The full length transcript was the most prominent and was inducibly stable The two other RNAs corresponded to RNAs terminating at either of the sequences encoding the 3' terminus of the 0 6 kb RNA As can be seen, the transcript corresponding to the downstream insert was more intense than that of the upstream insert I believe that this result strongly implies that the 0 6 kb RNA is a product of post-transcriptional processing and not of transcriptional termination If the 0 6 kb RNA was a product of transcriptional termination then the amount of RNA terminating in the upstream inserted sequence should be greater or equal to the amount terminating in the downstream inserted sequence since transcriptional termination should be equally efficient at both sites If the 3' end of the 0 6 kb RNA was produced by a blocking of RNA processing in the 3'-to-5' direction, then the amount of RNA corresponding to the downstream insert should be greater than the amount corresponding to the upstream insert The result seen in Figure 15 suggest that the 3' terminus of the 0 6 kb RNA was produced by processing The 0 6 kb RNA of BE253 and BE292 should be the same as the 0 6 kb RNA of BE8, therefore, the observation that this RNA is not dramatically stabilized in BE253 and BE292, but is in BE8 was surprising If this RNA was a product of RNA processing, the difference in stability may be caused by a difference in the initiation of decay of the downstream sequences

Figure 12. S1 analysis of the 3' terminus of the 0.6 kb RNA. The two probes used in the S1 analysis were the *lacZ BamHI-HpaI* fragment and the *lacZ BamHI-ClaI* fragment (Figure 7) labeled at their *BamHI* ends. S1 nuclease protection assays of the RNA from lanes 1 and 4, BD170 (no plasmid), lane 2, BE8 the time of rifampicin addition in the presence of Em; lanes 3 and 5, BE8 at 40 minutes after rifampicin addition, lanes 6 and 7, no RNA. The four lanes to the right are an M13mp19 DNA sequenceing ladder used as a size marker.

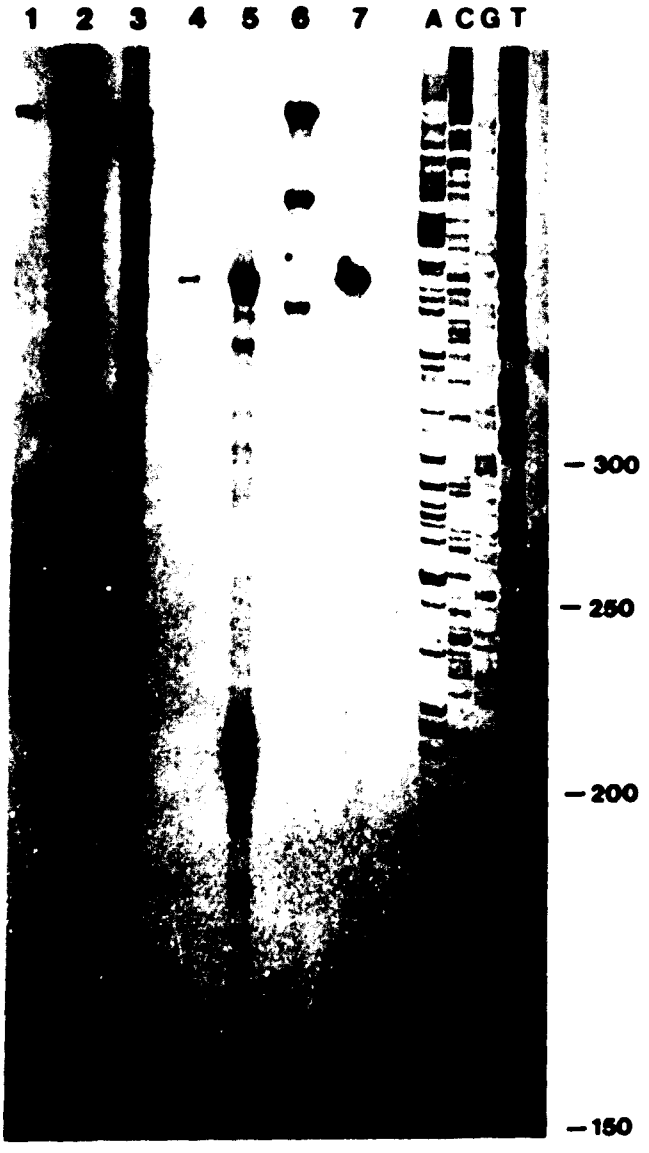
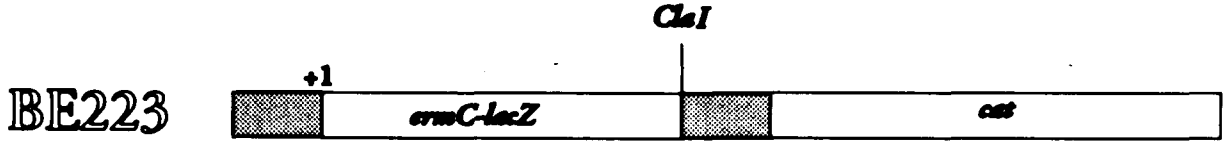

1 2 3 4 5 6 7 A C G T
300
250
200
150

Figure 13 Schematic of the *Cla*I deletions of pBD246 The sequences corresponding to the *erm*C'-*lacZ* and *cat* genes are indicated by open bars Sequences corresponding to extragenic plasmid sequence is represented by the filled bars The *Cla*I site of the *erm*C'-*lacZ* fusion is indicated The direction of transcription for each gene is indicated by the arrows BE223 contained the *cat* gene in the same orientation as the *erm*C'-*lacZ* fusion BE224 contained the *cat* gene transcrbed in the opposite direction as *erm*C'-*lacZ*


BE223
ClaI
+1
ermC-lacZ
cat

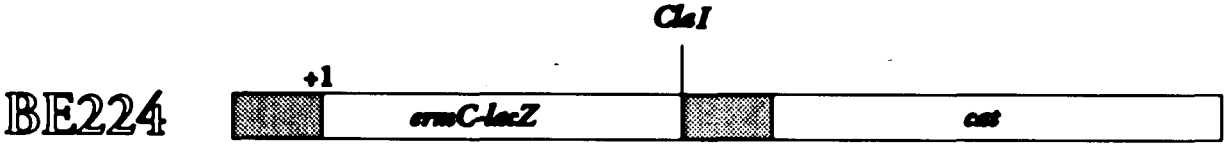

BE224
ClaI
+1
ermC-lacZ
cat

Figure 14 Schematic diagram of the plasmids pSD191 and pSD229 These plasmids contained either a single insert of the *lacZ* fragment that encoded the 0 6 kb 3' end (pSD191) or tandem copies of a *lacZ* fragment encoding this sequence (pSD229) The position and relative amounts of RNAs encoded by these plasmids is represented by the location and thickness of the lines below the construct

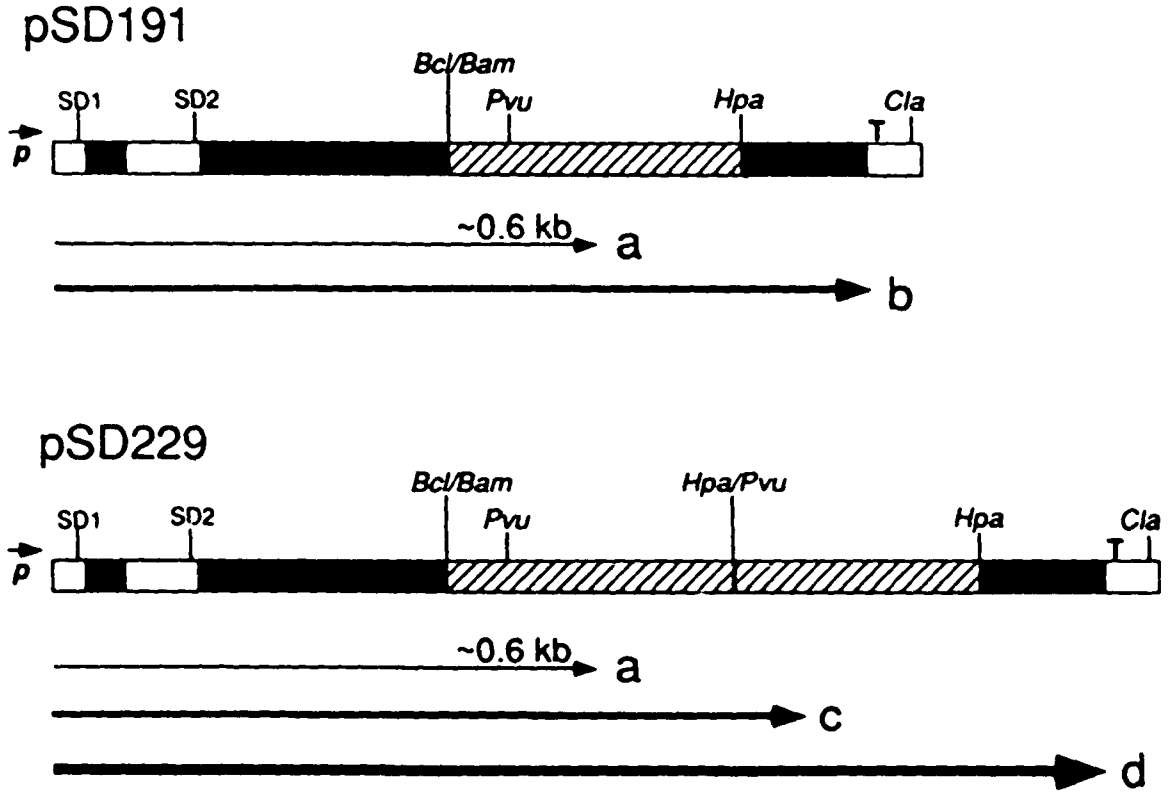
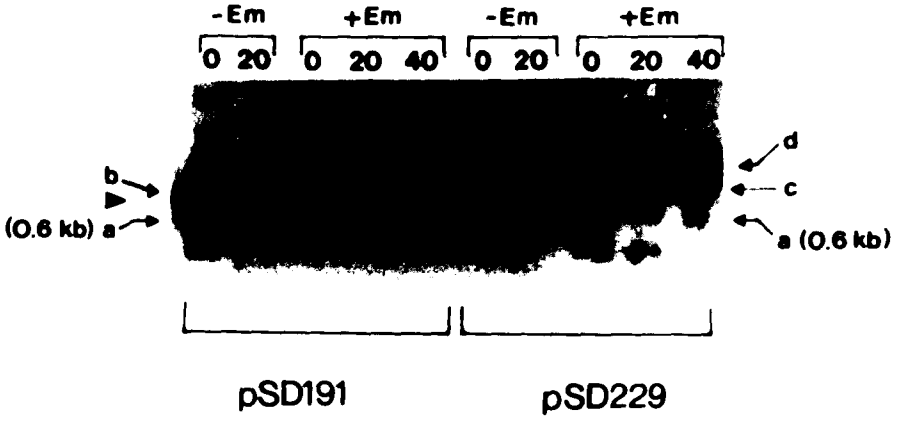

pSD191
Bcl/Bam
SD1
SD2
Pvu
Hpa
Cla
p
~0.6 kb
a
b
pSD229
Bcl/Bam
Hpa/Pvu
SD1
SD2
Pvu
Hpa
Cla
p
~0.6 kb
a
c
d

Figure 15 Northern blot analysis of RNA isolated from strains BE253 (pSD191) and BE292 (pSD229) Samples were taken from parallel cultures, grown in the absence (-) and presence (+) of Em, at various times after the addition of rifampicin The 0 6 kb RNA is indicated and the letters correlate to those in Figure 14


-Em
+Em
-Em
+Em
0 20
0 20 40
0 20
0 20 40
b
(0.6 kb) a
d
c
a (0.6 kb)
pSD191
pSD229

3.3 Insertion of an Endonucleolytic Cleavage Site into the *ermC* Coding Sequence

The ability of the 5' regulatory region of the *ermC* transcript to impart stability to diverse downstream sequences in the presence of Em-bound ribosomes has been demonstrated. One possibility for this result is that the initiation of decay in *B. subtilis* occurs exclusively at the 5' terminus of the transcript and that protection of the 5' terminus renders the transcript resistant to RNase activity. Alternatively, initiation of decay may occur internally but I may have been fortuitous in constructing *ermC* fusions with downstream sequences that lacked target sites for the initiation of mRNA decay. The insertion of an endonucleolytic cleavage site downstream of the *ermC* leader region might help to determine whether entry at the 5' terminus is an obligatory step in the initiation of decay. If an RNA containing an internal cleavage site was not inducibly stable in the presence of Em-bound ribosomes, it would indicate that the initiation of mRNA can occur at sites other than the 5' terminus in *B. subtilis*.

3.3.1 Insertion of a rRNA Spacer Sequence into the *ermC* Transcript

RNase III cleaves RNA duplexes in *E. coli*. This enzyme is involved in the maturation of transcripts from ribosomal operons, and cleaves the 30S rRNA at sites flanking the 16S and 23S rRNAs. It has also been shown to function in the initiation of decay of some *E. coli* mRNAs (see Background). An enzyme was isolated from *B. subtilis* that had a specificity for RNA substrates similar, but not identical to, those of *E. coli* RNase III (Panganiban and Whiteley,1983b). This enzyme has been purified and has been given the name Bs-RNase III. Comparison of the sequence of the

ribosomal operons of *E. coli* and *B. subtilis* demonstrated a high degree of homology (Green *et al*.,1985) Since RNase III processes ribosomal operon transcripts in *E. coli*, and since the *B. subtilis* rRNA sequence is similar to that of *E. coli*, the ability of Bs-RNase III to cleave in the 16S-23S spacer region of RNA transcribed from an rRNA operon of *B. subtilis* was tested (Paganiban and Whiteley,1983b). Bs-RNase III was found to cleave the spacer region of the rRNA

Insertion of a DNA fragment containing a Bs-RNase III target site into the *ermC* coding sequence might yield a transcript that could act as a Bs-RNase III substrate. If this transcript were cleaved, it would indicate that Bs-RNase III may play a role in mRNA decay in *B. subtilis*. Instability of this mRNA in the presence of Em-bound ribosomes would also demonstrate that the initiation of mRNA decay can occur at a site other than the 5' proximal region in *B. subtilis*. Therefore, I inserted a DNA fragment containing the 16S-23S ribosomal spacer region into the *ermC* coding sequence.

The plasmid p14B8 contained one complete but not intact *B. subtilis rrnG* ribosomal operon (Stewart *et al*.,1982). Internal to this sequence was a 520 bp *Sma*I fragment containing the 3' proximal 160 bp of the 16S rRNA gene, the spacer region, and the first 190 bp of the 23S rRNA (Figure 16). Plasmid p14B8 was digested with *Sma*I and this fragment was cloned into the *Hpa*I site of the *ermC* gene (Figure 17). The *Sma*I fragment was also cloned into the unique *Hinc*II site of M13mp7 for future subcloning.

Strain BE273 hosted plasmid pSD210 (Figure 17). Plasmid pSD210 contained the *Sma*I fragment of p14B8 inserted into the *Hpa*I site of *ermC* so that it was transcribed in the same orientation as the *rrnG* operon. RNA was isolated from strain BE273 grown in

the presence and absence of Em. Northern blot analysis was performed and the results of this analysis are shown in Figure 18A. Two bands were detected using a 5' *ermC'* probe: a 1.4 kb transcript, which was expected (the *Sma*I fragment is 520 nucleotides and the *ermC'* transcript is 910 nucleotides in length) and a larger transcript, which was unexpected. The 1.4 kb transcript was the more prominent of the two transcripts and both were found to be stabilized by Em-bound ribosomes. The half-life of these transcripts increased significantly upon Em induction demonstrating that 5' proximal protection of these RNAs by stalled ribosomes conferred stability.

Strain BE274 hosted plasmid pSD211, which contained the *Sma*I insert in such an orientation that this sequence was transcribed as the reverse complement of the *rrnG* transcript (Figure 17). Northern blot analysis was performed on RNA isolated from strain BE274 in the presence and absence of Em and the results of this analysis are shown in Figure 18A. Several RNAs were detected using an *ermC'* 5' probe including major bands corresponding to the expected 1.4 kb transcript and the longer transcript detected in BE273 RNA. Several other RNAs were detected that were longer than 1.4 kb and the higher band detected for BE273 RNA. The level of read-through past the *ermC'* transcriptional terminator was higher for BE274 than for BE273. Northern blot analysis of BE274 RNA demonstrated that the *ermC'-rrnG-ermC'* RNAs of this strain were not stabilized by Em and that these transcripts could only be detected at the zero time point of rifampicin addition.

The results of the Northern analysis of BE273 and BE274 RNA were surprising. If the inserted *rrnG* spacer sequence was acting as a Bs-RNAse III cleavage site, then

cleavage would have occurred in the *ermC'-rrnG-ermC'* fusion of BE273, which contained the same target sequence as the ribosomal operon However, in BE273, the effect of the insert was only antitermination Possible explanations for the antitermination are described in the Discussion section of this thesis The instability of the *ermC'-rrnG-ermC'* RNAs in BE274 was unexpected since they did not encode the Bs-RNase III cleavage site, but a reverse complement of this sequence The fact that instability was seen for RNA in BE274, but not in BE273, suggested that degradation of the RNAs was occurring by some mechanism other than the recognition the Bs-RNase III cleavage site The instability of the BE274 RNAs could be due to an effect that this insert had on translation or to some change in the secondary structure produced by this insert The appearance of unexpected lengthy transcripts indicated that this insert produced antitermination This effect was more dramatic in the *ermC'-rrnG-ermC'* construct of BE274 which was transcribed to give the reverse complement of the ribosomal transcript

The possibility that the sequence of the *rrnG* spacer region acts as an endonucleolytic processing site when transcribed as the reverse complement was tested by Northern blot analysis using both a 3' and 5' *ermC'* probe While there was no difference in the pattern of the transcripts detected with either probe from RNA isolated from strain BE273, the two probes revealed different bands upon analysis of BE274 RNA (Figure 18B) A small and a large RNA species were detected with the 3' probe that were not detected with the 5' probe The small RNA may represent a downstream cleavage product generated by cleavage of the full-length RNA This result suggests that the reverse complement of the rRNA spacer region functioned as an endonucleolytic cleavage site

The large RNA that was detected in BE274 may be the product of processing of the RNAs produced by read-through past the *ermC'-rrnG-ermC'* transcriptional terminator The Northern blot of BE274 RNA using the 3' *ermC'* probe was problematic, however, since the 1 4 kb full-length *ermC'-rrnG-ermC'* transcript was barely visible There should have been no difference in the relative intensities of this band in blots probed with the 3' and 5' probes Nevertheless, the presence of the small RNA was consistent with the conclusion that the rRNA spacer sequence acted as an endonucleolytic cleavage site when inserted in the opposite orientation as it exists in the rRNA operon These results also demonstrated that the initiation of decay of *B. subtilis* transcripts can occur at sites other than the 5' terminus since transcripts containing this sequence were unstable even with stalled Em-bound ribosomes protecting the 5' terminus from attack

The possibility that the difference in cleavage of RNA in BE273 and BE274 may have been due to differences in translation was tested Northern analysis was performed on RNA isolated from strains identical to BE273 and 274 except that the SD2 sequence of these *ermC'-rrnG-ermC'* fusions was non-functional The decay pattern of RNA isolated from these strains were the same as for BE273 and BE274 (data not shown)

It was possible that the loss of induced stability of transcripts from plasmid pSD211 was due to some secondary structure that formed between the *ermC'* sequence and the inserted *rrnG* sequence To address this, the same fragment of *rrnG* was inserted into a different location of the *ermC'* coding sequence As was mentioned above the *SmaI* fragment of p14B8, which contained the 16S-23S spacer region, was inserted into the *Hinc*II site of M13 mp7 which is flanked by two *Bam*HI sites RFII phage DNA

was isolated from JM109 cells infected with this construct and the DNA was digested with *Bam*HI. The *Bam*HI fragment was isolated and cloned into the *Bcl*I site of the *ermC'* methylase coding sequence (Figure 7). Strains were isolated which contained the *Bam*HI fragment cloned in the same orientation as the *rrnG* operon (BE279) and in the opposite orientation (BE280). Northern blot analysis was performed on RNA isolated from these strains and the same results were seen as in BE273 and BE274. The RNA isolated from BE279 was inducibly stable in the presence of Em while transcripts of BE280 were not inducibly stable (data not shown). The production of longer transcripts was also detected in these strains and as before the effect was greater in the strain which contained the inverted sequence (BE280). These results demonstrate that the effects of these *rrnG* sequences are not dependent on the sequence flanking these inserts since the same effect is demonstrated regardless of where the insertion is made in the *ermC'* gene.

The insertion of the *rrnG* spacer region into a sequence other than *ermC'* would allow us to examine the effect of this insert in another context. The rRNA spacer region was cloned in both orientations into the *lacZ* coding sequence of the *ermC'-lacZ* fusion. The rRNA spacer region was inserted at a *Bcl*I site 1,100 base pairs downstream of the sequence encoding the 3' terminus of the 0.6 kb RNA of pBD246. Northern analysis was performed on RNA isolated from strain BE290 which contained the rRNA spacer region inserted in the same orientation as the ribosomal operon (Figure 19). Three major bands were detected using an *ermC'* 5' probe: a 0.6 kb RNA, which corresponded to the 0.6 kb RNA of the *ermC'-lacZ* fusion, and two transcripts that corresponded to the 4 and 6 kb RNAs plus the 520 nucleotide insert. Both the 4.5 and 0.6 kb transcripts were inducibly

stable in the presence of Em while the 6 5 kb transcript was not These results agree with those expected from the Northern analysis of BE273 in which transcripts containing the rRNA sequence were inducibly stable

Northern blot analysis of RNA isolated from the strain which contained the insert in the reverse orientation as the ribosomal operon (BE291) is shown in Figure 19 These results demonstrated that this inserted sequence has the same effect in the *BclI* site of pBD246 as it did in the *HpaI* site of pSD211 There was a notable degree of antitermination as was evident by the presence of transcripts larger than the longest expected transcript of 6 5 kb Again it was demonstrated that insertion of this sequence resulted in a loss of induced stability Here, however, the loss of induced stability only occurred with the 4 5 kb transcript The 0 6 kb RNA was still inducibly stable, as in strain BE8

Figure 16 Schematic diagram of the *B. subtilis rrnG* ribosomal RNA operon The 520 base pair *Sma*I fragment is indicated

B. subtilis rrnG
ribosomal RNA operon

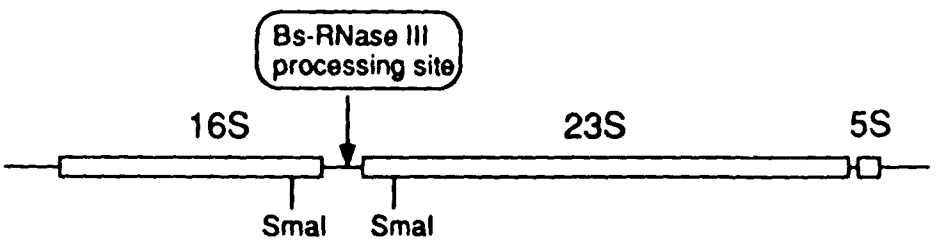


Figure 17 Schematic of the insertion of the *Sma*I fragment of the *rrnG* ribosomal rRNA operon into the *Hpa*I site of the *ermC* gene The *rrnG* fragment is represented by the stippled bar The orientation of this fragment in the *rrnG* operon is represented by the arrow The promoter (P) and terminator (T) sites for *ermC* are shown BE273 contained the *Sma*I insert in the same orientation as the *rrnG* operon and BE274 contained the *Sma*I insert in the opposite orientation as the *rrnG* operon

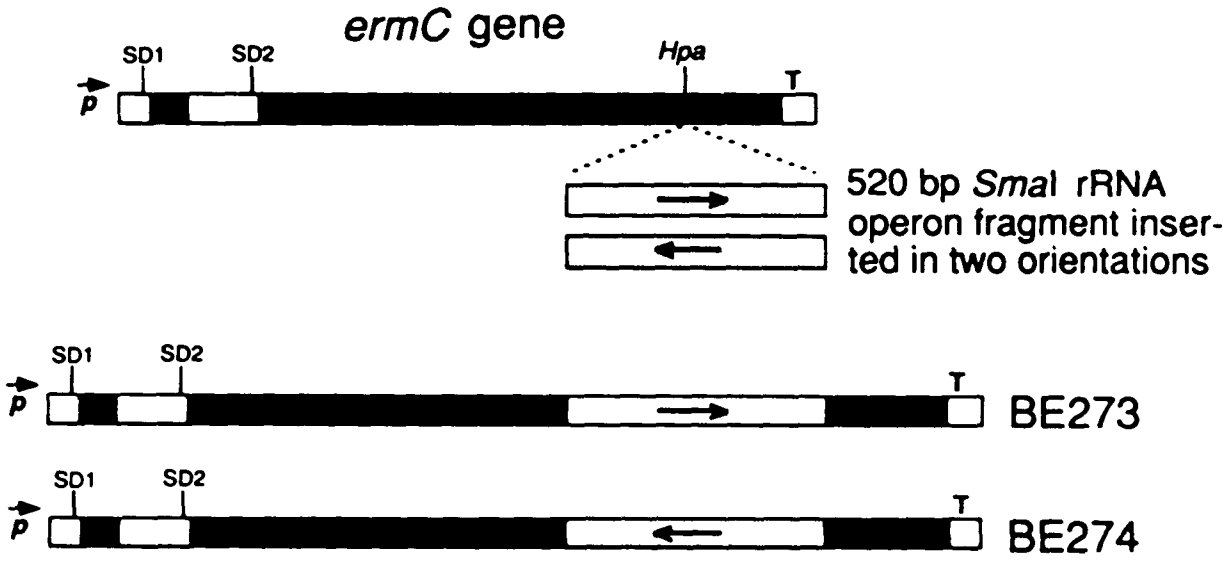
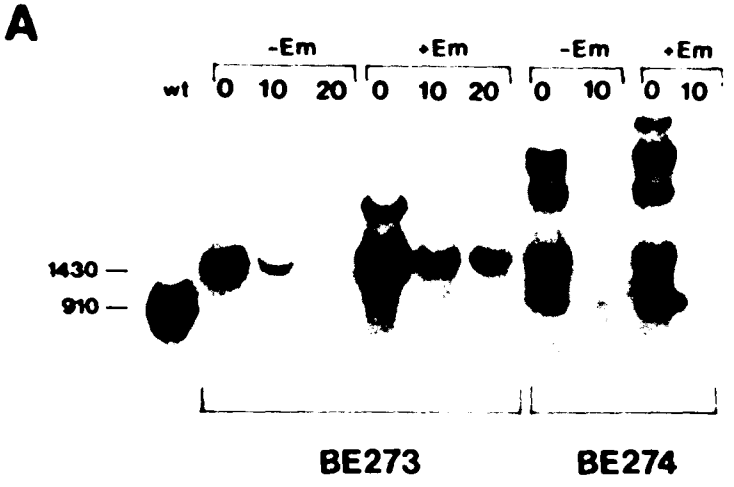

ermC gene
SD1
SD2
Hpa
T
P
520 bp SmaI rRNA operon fragment inserted in two orientations
SD1
SD2
T
P
BE273
SD1
SD2
T
P
BE274

Figure 18 Northern blot analysis of RNA isolated from strains BE273 and BE274 (A) The time (in minutes) after rifampicin addition is indicated above each lane The numbers to the left of the blots are the size of the wild-type *ermC'* mRNA and the predicted size of the *ermC'* RNA containing the 520 nucleotide insert The *ermC'* 5' probe was used for this analysis (B) Northern blot analysis of RNA isolated from strains BE273 and BE274 using a 5' and a 3' *ermC'* probe on the same blot


A
-Em
+Em
-Em
+Em
wt
0
10
20
0
10
20
0
10
0
10
1430
910
BE273
BE274

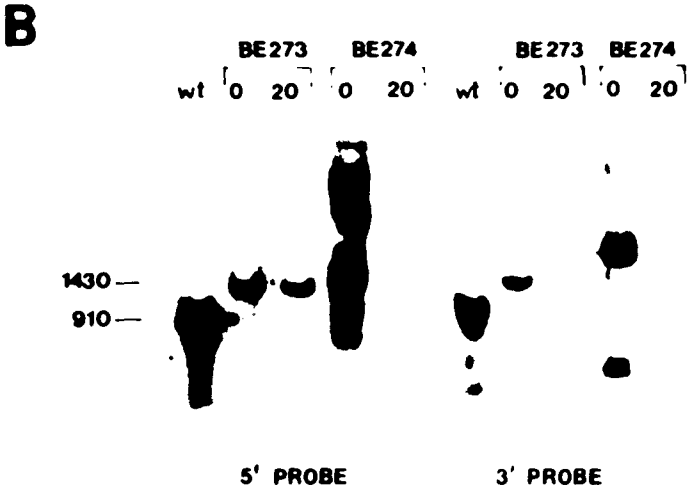
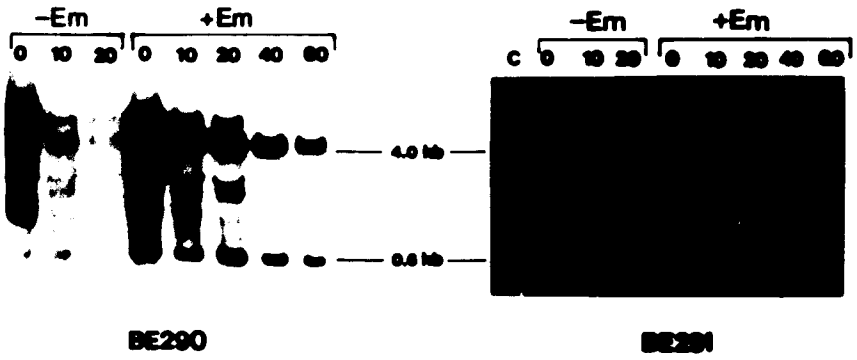

B
BE273
BE274
BE273
BE274
wt
0
20
0
20
wt
0
20
0
20
1430
910
5' PROBE
3' PROBE

Figure 19. Northern blot analysis of RNA isolated from strains BE290 and BE291. BE290 contained an *ermC'-lacZ* fusion with the *rrnG* fragment inserted into the *BclI* site of *lacZ* (see Figure 7) in the same orientation as the *rrnG* operon. BE291 contained an *ermC'-lacZ* fusion with the *rrnG* fragment inserted into the *BclI* site of *lacZ* in the opposite orientation as the *rrnG* operon. Samples were taken in the absence (-) and presence (+) of Em. The time (in minutes) after the addition of rifampicin is indicated above each lane. BE8 RNA was run as a size marker control.


-Em
+Em
0 10 20 0 10 20 40 60
4.0 kb
0.6 kb
BE290
-Em
+Em
C 0 10 20 0 10 20 40 60
BE291

3.4 Insertion of an SP82 Bs-RNase III Site into the *ermC'* Coding Sequence

The fusions containing the *rrnG* spacer region demonstrated that the initiation of mRNA decay could occur at sites other than the 5' proximal region of a message The complication that the loss of stability only occurred when the reverse complement was transcribed and that antitermination was occurring led us to search for another endoribonucleolytic cleavage site SP82 is a bacteriophage of *B. subtilis* that has a 140 kbp genome It has been demonstrated that early RNAs transcribed from the left terminal repeat of bacteriophage SP82 are cleaved *in vivo* and that this cleavage could be reproduced *in vitro* using a purified Bs-RNase III (Panganiban and Whiteley,1983a) A Bs-RNase III cleavage site from bacteriophage SP82 was cloned into *ermC'* and the effects on mRNA stability were analyzed

3.4.1 Cloning of a Bs-RNase III Site into *ermC'*

The left terminal repeat of SP82 contains three Bs-RNase III cleavage sites in the first 1 4 kbp of sequence (Figure 20) An SP82 transcriptional promoter exists upstream of these Bs-RNase III sites It has been found that the left terminal repeat cannot be cloned in *E. coli* or *B. subtilis* presumably due to this promoter (Hemphill, personal communications) Therefore, cloning of the entire 1 4 kbp left-most sequence was not desired Attempts were made at cloning DNA fragments that encode Bs-RNase III cleavage sites into the *ermC'* coding sequence A 7 5 kbp *Hae*III fragment was isolated that contained the left end 7 5 kbp of the SP82 genome Attempts were made to isolate a 110 base pair *Sca*I-*Hpa*I fragment from the *Hae*III fragment but these were unsuccessful

because digestion of this fragment was inefficient and we could not obtain a sufficient quantity of the *Sca*I-*Hpa*I fragment for cloning. This may have been due to the nature of SP82 DNA which is modified and contains hydroxymethyluridine instead of thymidine. In another attempt, the terminal 1.4 kbp fragment of SP82 DNA was isolated after digestion with *Hae*II. This fragment was digested with *Rsa*I since one of the Bs-RNase III cleavage sites was flanked by *Rsa*I sites. Again the yield of this *Rsa*I fragment was poor. I attempted to clone this *Rsa*I fragment into the *Hpa*I site of *ermC'*, but no colonies containing this insert were found after transformation of BG1 with this DNA. In order to obtain a high concentration of a DNA fragment encoding a Bs-RNase III site, the polymerase chain reaction was employed to amplify a sequence encoding a Bs-RNase III site that did not contain SP82 transcriptional signals. Primers were used that contained *Bam*HI recognition sequences so that the PCR amplicon could be cloned into the *Bcl*I site of *ermC'* in two orientations (Figure 20). The sequence of the 370 base pair amplicon is presented in Figure 21.

Strain BE301 contained the insert in the orientation such that transcription of this insert produced the same RNA sequence as SP82 RNA. Strain BE303 contained the insert in the opposite orientation so that transcription of this sequence produced the reverse complement of the SP82 RNA sequence. Northern blot analysis was performed on total RNA isolated from strains BE301 and BE303 in the presence and absence of Em at times after the addition of rifampicin. The results of Northern blot analysis of RNA isolated from strain BE301 using a 5' *ermC'* probe are shown in Figure 22A. The expected full-length transcript of this fusion should be 1,280 nucleotides. Only a faint band of the expected size

was detected with the 5' probe, which indicated that the cleavage of this RNA was efficient. The major RNA species that was detected with this probe was about 600 nucleotides in length and was not inducibly stable in the presence of Em. Northern blot analysis of this RNA was then performed using a 3' *ermC* probe and the results are shown in Figure 22B. The 3' probe detected an RNA which was approximately 600 nucleotides in length and which was stable in both the presence and absence of Em. These results were consistent with the inserted sequence acting as a target site for Bs-RNase III cleavage. Cleavage at the defined Bs-RNase III cleavage site would produce two RNAs of approximately 600 nucleotides each. The stability of the downstream cleavage product was intriguing since it demonstrated that an mRNA can remain stable after cleavage, despite having an unprotected 5' end.

A model for the processing of the BE301 transcript is shown (Figure 23A). A stem-loop structure forms in the RNA that is a Bs-RNase III cleavage site. The presence of an Em-bound ribosome in the 5' leader region does not confer stability to this transcript, indicating that Bs-RNase III does not associate with the 5' terminus prior to cleaving internally. After cleavage the upstream fragment is rapidly degraded while the downstream fragment remains stable. The upstream cleavage product is most likely degraded by 3'-to-5' exonucleases that can access the 3' terminus of this RNA. The reason for the stability of the downstream cleavage product is discussed later.

Northern blot analysis was performed on RNA isolated from strain BE303, which contained the SP82 DNA insert in the opposite orientation such that transcription of this sequence produced the reverse complement of SP82 RNA. Both the 5' and 3' *ermC*

probes were used and the results are shown in Figure 22C and D The expected full-length RNA was readily detected with both the 5' and 3' *ermC'* probes and this full-length transcript was found to be inducibly stabile in the presence of Em, unlike the full length RNA isolated from BE301 The 5' *ermC'* probe revealed a smaller RNA species which was also inducibly stable in the presence of Em This RNA was approximately the same size as the cleavage products of BE301 A downstream cleavage product could not be detected using the 3' *ermC'* probe, which only detected the full-length transcript These results demonstrated that transcripts of the construct contained in BE303 were cleaved and that only the upstream cleavage product remained stable after cleavage since only this product could be detected The quantity of full length transcript detectable in BE303 compared to that of BE301 suggested that while cleavage did occur in the strain expressing the reverse complement of the Bs-RNase III cleavage site, the cleavage was not as efficient as when the sequence was expressed in the same orientation as SP82

3.4.2 Mapping of the Cleavage Sites of BE301 and BE303 RNA

Mapping of the termini of the upstream and downstream cleavage products of BE301 RNA was performed so that the site of endonucleolytic cleavage could be defined Reverse transcription analysis was done to identify the 5' terminus of the downstream cleavage product of BE301 RNA using a primer that was complementary to sequences 260 to 246 of the inserted sequence To eliminate the possibility that a strong stop site for reverse transcription might be mapped, reverse transcription was also performed on *in vitro* transcribed RNA of this sequence The reverse transcription of both the *in vivo* and

in vitro RNAs was performed at 42° C (Figure 24, lanes 5,6,7,8), 45° C (lanes 3,4,9,10) and 50°C (lanes 1,2,11,12) to ameliorate premature termination of transcription of the reverse transcriptase due to secondary structure. A strong band was detectable in the lanes containing the reverse transcription reactions of RNA isolated from strain BE301 which was not detectable in the *in vitro* transcribed RNA lanes. This band was detected at all temperatures and corresponds to nucleotide 216 of the Bs-RNAse III sequence (Figure 21). I conclude from this data that the 5' terminus of the BE301 downstream cleavage product is at position 216 of the Bs-RNase III sequence and that this RNA is 700 nucleotides in length.

In order to determine the 3' terminus of the upstream cleavage product of BE301, S1 analysis was performed using a *Bam*HI-*Nsi*I probe end-filled at the *Bam*HI site. The restriction sites employed are shown in Figure 21. A doublet was detected by S1 analysis of RNA isolated from strain BE301 using this probe and the two bands corresponded to nucleotides 214 and 215 of the Bs-SP82 RNase III sequence (data not shown). These bands map precisely to the cleavage site determined by reverse transcriptase mapping of the 5' terminus of the downstream cleavage product. I conclude from this data that the downstream cleavage product of BE301 was 580 nucleotides in length. The fact that the 3' terminus of the upstream cleavage product and the 5' terminus of the downstream cleavage product of BE301 mapped to adjacent nucleotides demonstrated that post-cleavage processing did not occur.

The 3' terminus of the upstream cleavage product of BE303 RNA was also mapped by S1 analysis (data not shown). The probe for this analysis was a *Taq*I- *Bam*HI

fragment end-filled at the *Bam*HI site The 3' terminus was found to map at position 134 of the Bs-RNase III sequence (numbering used is for position of sequence in same orientation as SP82) and is indicated by an asterisk (Figure 21) The upstream cleavage product of BE303 was therefore about 595 nucleotides in length

The results from the mapping experiment indicated that the upstream and downstream cleavage products of BE301 should have been 580 and 700 nucleotides in length, respectively The size of the upstream cleavage product of BE303 should have been 595 nucleotides in length (schematic, Figure 25) Northern blot analysis was performed on RNA isolated from BE301 and BE303 These RNAs were loaded side by side along with RNA size markers and run for a longer time than for the above Northerns to increase separation The blots were probed with either a 5' or 3' *ermC* probe (data not shown) The migration of these RNAs agreed with the estimated sizes from mapping of the termini of these RNAs

Figure 20 Schematic diagram of the left-most 1 4 kbp of bacteriophage SP82 The sequences encoding the RNase III cleavage sites are indicated by the filled boxes The putative promoter is shown as well as the restriction sites used for cloning The sequence corresponding to the PCR amplicon is indicated by the black bar under the diagram

Left Terminal Repeat of SP82
First 1.4 kbp

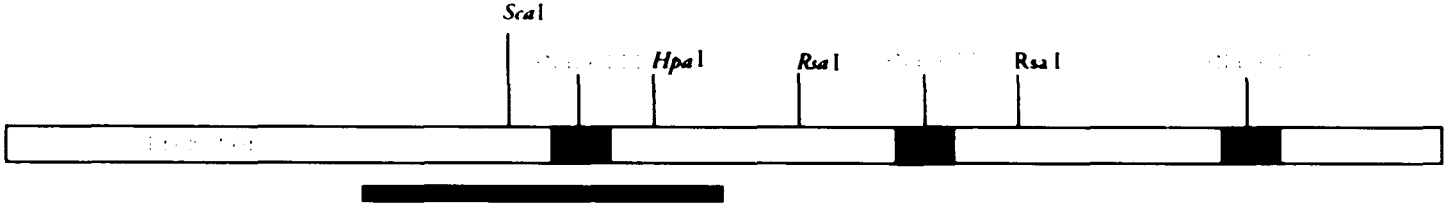


Figure 21 Sequence of the *Bam*HI fragment encoding a Bs-RNase III site from bacteriophage SP82 This sequence is for the *Bam*HI fragment in the same orientation as in SP82 The Bs-RNase III recognition sequence is underlined The mapped cleavage site is indicated by the single tailed arrow The cleavage site mapped by Panganiban and Whiteley (1983,a) is indicated by the double tailed arrow Point mutations are indicated by lower case letters The non-SP82 sequence produced by PCR is indicated by the small capital letters The potential ribosome binding site is shown (RBS) Pertinent restriction sites are shown The sequence complimentary to the primer used for the reverse transcription analysis is shown in bold letters

*Bam*HI *Taq*I

5'-GGATCCCGCAACCTAGCAACGCACCAGTAATCGAGGTATTCACAGAGGAT

gATCTTGAAGAGGGTATCATCCCTGAGTATGTTACCGCCAACGATGATAC

ATTTGACCGTATCGTAGACGCTGTAGAGTTTGGC*TATCTGGAAGGACTGG

AGCTAGTATAGGCTCCAGTCCCGCTTCAGCAGTTGCTAGTACTCACCCA⇓A

*Alu*I RBS

GCGGGTGGGCACCAT↓GGAGCCGCTGAGCTACCACAGATTGTgAAA**GGAGA**

*Hpa*I

aGTTAACATGAAAAAACGTTTAAAGGTAACACCATTATTTGAGGCTGGTACATCACAGTGTTTAGTG

*Nsi*I *Bam*HI

GTAGGAATTTTTCTTCCCCTACAAATGCATGGTGTGCCGTTATACAAGCGGATCC-3'

Figure 22 Northern blot analysis of *ermC'* constructs with the inserted SP82 sequence BE301 contained the SP82 insert in the orientation such that it was transcribed in the same direction as SP82 DNA BE303 contained the SP82 sequence such that it was transcribed to give the reverse compliment as the SP82 RNA BE301 RNA was probed with the 5' and 3' *ermC'* probes (A and B respectively) as was the BE303 RNA (C and D) The time (in minutes) after rifampicin addition is indicated above each lane Numbers on the left side are the sizes of the RNAs (in nucleotides) that were detected Marker lanes are E, *ermC'* wild-type (910 nt), and EP, *ermC'-penP* fusion (400nt)

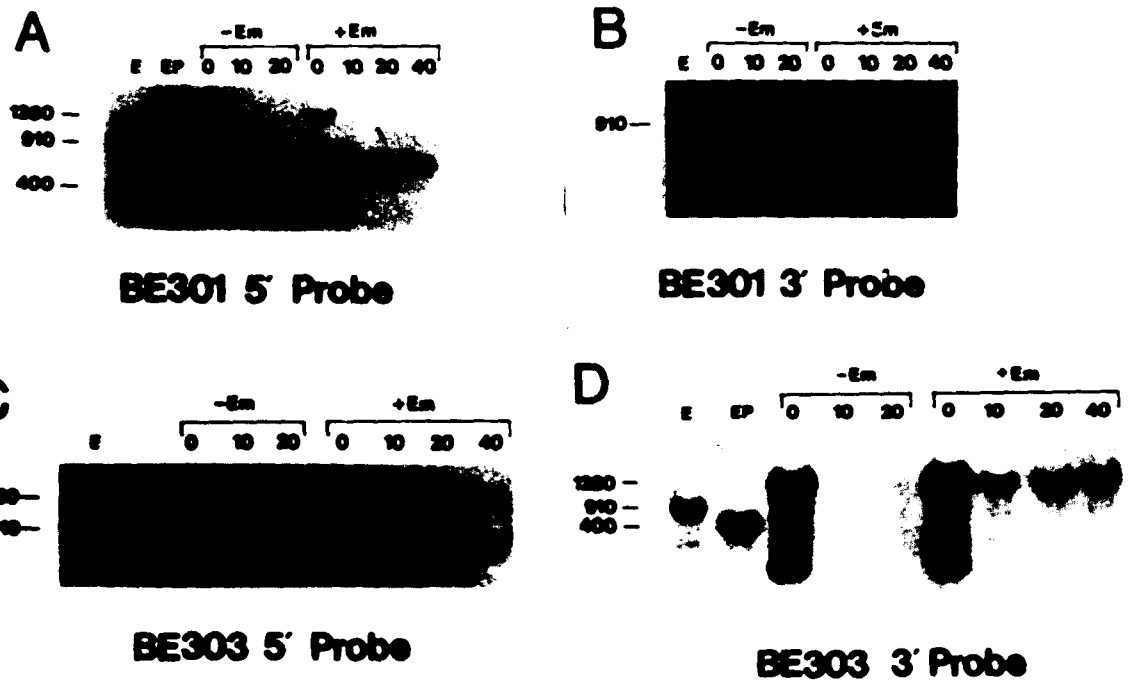
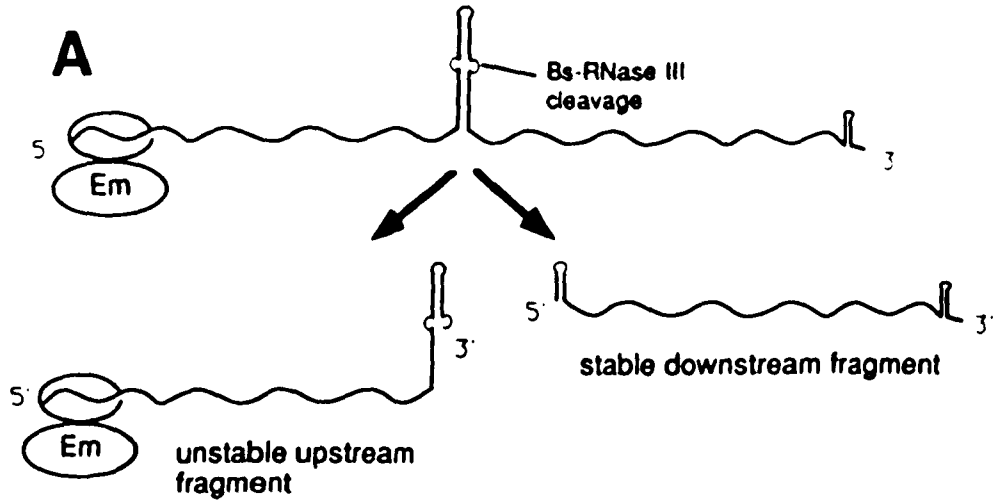
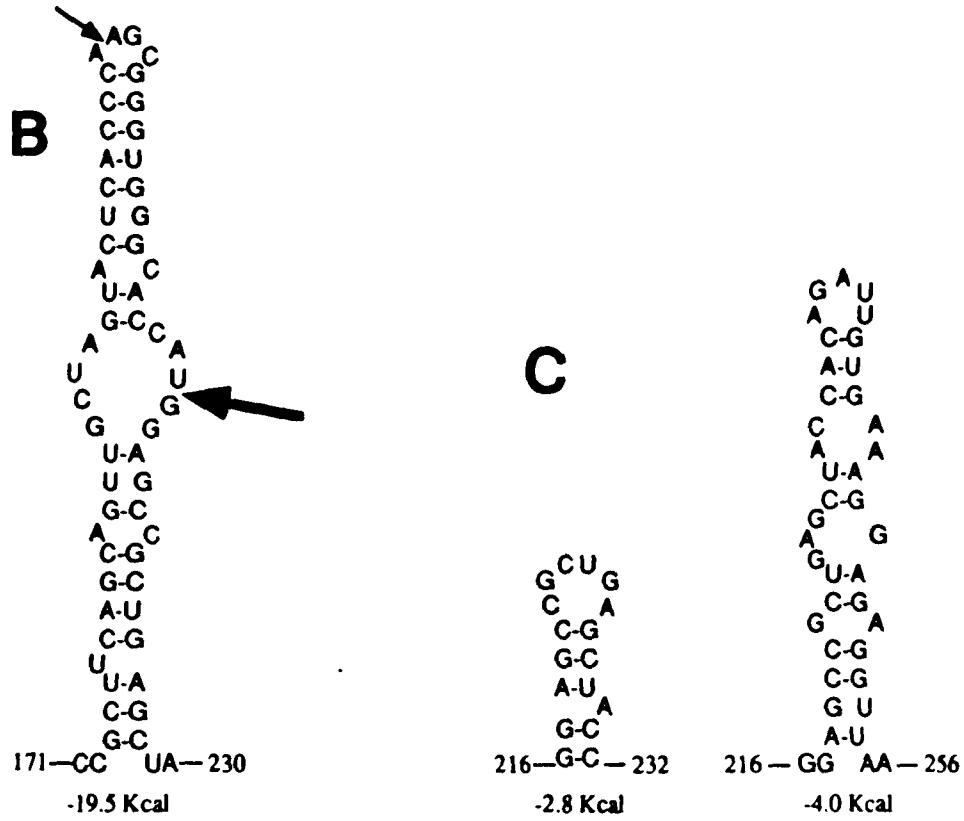
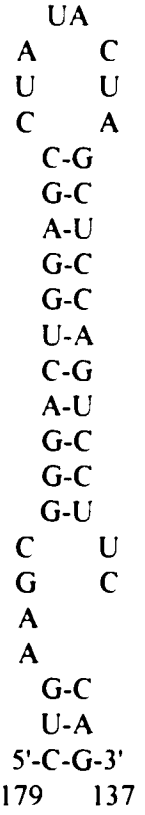

A
-Em
+Em
E EP 0 10 20 0 10 20 40
1280
910
400
BE301 5' Probe
B
-Em
+Em
E 0 10 20 0 10 20 40
910
BE301 3' Probe
C
-Em
+Em
E 0 10 20 0 10 20 40
BE303 5' Probe
D
-Em
+Em
E EP 0 10 20 0 10 20 40
1280
910
400
BE303 3' Probe

Figure 23 Bs-RNase III cleavages (A) Model for the generation of the processed RNA fragments of BE301 (B) Predicted secondary structure of the regions surrounding the Bs-RNase III cleavage site The thick arrow represents our mapped cleavage site, the small arrow indicates the site of cleavage mapped by Panganiban and Whiteley (1983,a) (C) Possible secondary structures of the 5'-terminal region of the downstream cleavage product of BE301 RNA The numbering corresponds to the sequence Figure 21 The free energy of each structure is given (D) Possible secondary structure for the 3'-terminal region of the upstream cleavage product of BE303 RNA The free energy of this structure is given


A
Bs-RNase III
cleavage
5
3
Em
5'
3'
stable downstream fragment
5'
3'
Em
unstable upstream
fragment


B
171—CC UA— 230
-19.5 Kcal
C
216—G-C— 232
-2.8 Kcal
216 — GG AA — 256
-4.0 Kcal

D


UA
A C
U U
C A
C-G
G-C
A-U
G-C
G-C
U-A
C-G
A-U
G-C
G-C
G-U
C U
G C
A
A
G-C
U-A
5'-C-G-3'
179 137

-19.5 kcal

Figure 24 Reverse transcriptase mapping of the BE301 RNA downstream cleavage product. The sequence of the primer used is indicated in Figure 21 (bold face). Control lanes (ACGT) are a DNA sequencing ladder using the same primer. Following annealing of the primer on *in vitro*-transcribed RNA (lanes 1-6) or total cellular RNA (lanes 7-12), reverse transcriptase was added and incubated for 30 minutes at 42° C (lanes 5,6,7,8), 45° C (lanes 3,4,9,10), or 50°C (lanes 1,2,11,12). Either 1 µl (lanes 2,3,6,7,10,11) or 5 µl (lanes 1,4,5,8,9,12) of a 20 µl reaction volume were loaded on the gel. Lane 13 is the labeled primer alone. The arrow indicates the band representing the 5' end of the downstream cleavage product of BE301 RNA.

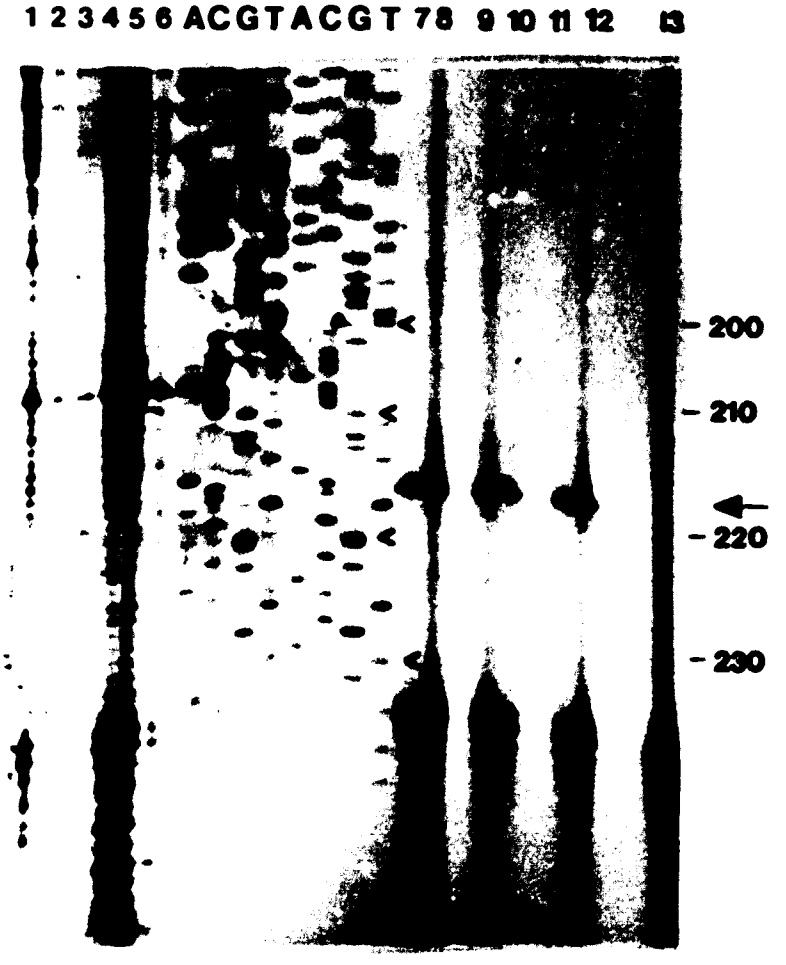

1 2 3 4 5 6 A C G T A C G T 7 8 9 10 11 12 13
200
210
220
230

Figure 25 Schematic of the cleavage products of BE301 and BE303 RNAs The *ermC* regions are represented by open bars and the SP82 insert by the stippled bars The orientation of the insert relative to SP82 is indicated by the arrows The *ermC* promoter (P), transcriptional terminator (t), and ribosome binding sites are shown (SD) RNAs are represented by the lines under the construct The fate of the RNA fragments after cleavage is shown

BE301

5' ——————————————— 3'

5' ——————— 3' 5' ——————— 3'
Unstable Stable

BE303

5' ——————————————— 3'

5' ——————— 3' 5' ——————— 3'
Inducibly stable Unstable

Chapter Four

Discussion

Discussion

The regulation of messenger RNA decay is an important element of the cellular mechanisms for the regulation of gene expression. The process by which the initiation of mRNA decay occurs is poorly understood. Here I have employed the *ermC'* transcript as a model to help in our understanding of the initiation of decay of *B. subtilis* transcripts. The *ermC'* transcript is an attractive model since it can be inducibly stabilized by the stalling of Em-bound ribosomes in its 5' regulatory region. This stalling renders the transcript resistant to degradation and a 15 to 20 fold increase in message stability has been noted (Bechhofer and Dubnau, 1987). Studies of the initiation of decay of transcripts in *E. coli* have demonstrated the importance of an accessible 5' terminus of the transcript for the initiation of RNA decay (Emory *et al*, 1992). To determine if 5' accessibility is the rate limiting factor in mRNA decay in *B subtilis*, I examined the decay of transcripts which contained the 5' regulatory region of *ermC'* fused to diverse downstream sequences.

The results of the transcriptional fusions (Figure 11) demonstrated that the 5' regulatory region of *ermC'* can confer stability to diverse downstream sequences. I conclude from this data that the 5' regulatory region of *ermC'* can act *in cis* as a "general stabilizer". The conclusion one can draw from these results is that inspection of the 5' terminus by some yet unidentified nuclease is the rate limiting step in the degradation of mRNAs in *B. subtilis*. If this were so, it would explain how a ribosome stalled in the 5' leader region of the *ermC'* transcript could impart stability to the entire transcript. A model has been proposed in which an RNase must first bind to the 5' proximal region of an mRNA in order for the initiation of decay to occur (Figure 1B). After binding, the RNase

migrates down the mRNA or loops to a downstream target site where it cleaves The RNA is then degraded in the 3'-to-5' direction In this model the initiation of decay occurs only when the 5' terminus is accessible, but the direction of decay is 3'-to-5' Such a hypothesis is supported by recent evidence that demonstrates that the initiation of decay of an otherwise unstable *E. coli* transcript can be hindered if the 5' terminus is sequestered in a stable stem-loop structure (Emory *et.al*, 1992) The directionality of decay was examined and it was found that the decay initiated internal to the body of the mRNA and not from the 5' terminus This was supported by an earlier study that demonstrated that this mRNA decayed with a 3'-to-5' directionality (von Gabain *et al*., 1983) The authors concluded that sequestering of the 5' terminus did not increase stability due to the blockage of a 5' exoribonuclease, but rather by blocking the action of an endoribonuclease that initiates decay by cleaving downstream, but which must first bind the 5' terminus.

The ability of this region to act as a stabilizer appears to depend on the downstream sequence since the fusions did not all possess the same half-lives in the presence of Em-bound ribosomes This suggests that the initiation of decay at the 5' terminus is the rate limiting step for the initiation of decay, however, it is not the only means for the initiation of decay The decay of RNA can initiate downstream, but this type of initiation occurs at a slower rate than the initiation at the 5' terminus.

The result of the *ermC'-penP* fusion was interesting since it appeared to conflict with previous results *penP* mRNA had a 2 minute half-life in *B. subtilis*, (Wong and Chang,1986). Replacement of the sequence encoding the 40 3' proximal base pairs of *penP* with the transcriptional terminator of the crystal protein (*cry*) increased the half-life

to 6 minutes, which was comparable to the half-life of the *cry* mRNA. The authors concluded that the 40 3' proximal nucleotides of *penP* were responsible for the short half-life of this transcript. In our study, the *ermC'-penP* transcript contained the 360 5' proximal nucleotides of *ermC'* and the 40 3' proximal nucleotides of *penP*. This mRNA was found to be inducibly stable in the presence of Em-bound ribosomes, suggesting that the 3' *penP* sequence was not the major determinant of stability. Why is the 3' sequence of the *penP* mRNA not recognized as a site for the initiation of decay in the *ermC'-penP* fusion? If the initiation of decay of mRNA in *B. subtilis* occurs with a 5'-to-3' directionality, the difference between our result and that of Wong and Chang could be explained. Although both the *penP* and *ermC'-penP* mRNAs contain the same 3' proximal sequence, the accessibility of their 5' regions is quite different. The *penP* transcript contains an accessible 5' region that can be bound by RNases, while the 5' region of *ermC'-penP* is not accessible due to the presence of stalled Em-bound ribosomes. If the RNase that cleaves the 3' proximal region of *penP* must first bind the 5' region of the message before it can recognize the downstream cleavage site, then the *penP* transcript with an accessible 5' region would be degraded and the *ermC'-penP* transcript would remain stable.

The 5'-binding nuclease that initiates the degradation of RNAI has recently been identified and it is RNase E (Bouvet and Belasco, 1992; see Background). Dr. Bechhofer has searched for an analog of RNase E in *B. subtilis* by Southern blotting with a probe containing the *E. coli* RNase E gene. No bands were detected using this probe.

The results of the *ermC'-lacZ* fusion demonstrate that there are limitations to the stabilizing effect of the 5' regulatory region of *ermC'* The 6 kb transcript of this fusion is not inducibly stable in the presence of Em-bound ribosomes (Figure 8) This may be due the presence of endonucleolytic cleavage sites or the nature of the 3' proximal sequence of the transcript Since I have not examined this RNA, any attempt to explain its short half-life would be speculation The 4 kb transcript may be the product of transcriptional termination at the normal *lacZ* terminator The *ermC'-lacZ* fusion contains the first 360 nucleotides of *ermC'* fused to approximately 3,200 nucleotides of *lacZ* sequence Murakawa *et al* have reported that a rho-independent transcriptional terminator exists in the *lacZ-Y* intergenic spacer region (Murakawa *et al* , 1991) If there was a cessation of transcription at the normal terminator, the expected transcript would be about 3 5 kb in length The "4" kb transcript may actually be this transcript, but this remains unknown since the 3' terminus of this transcript has not been mapped If this were the case, however, it might explain why this transcript is inducibly stable since this transcript would be a product of rho-independent termination and would contain a 3' secondary structure that could protect the 3' terminus from RNase attack

The results of my analysis indicated that the 0 6 kb RNA was a product of RNA processing The pattern of RNA bands seen in the Northern analysis of BE292 RNA (Figure 14), which contained the tandem repeats of this sequence, supports this conclusion The presence of a smear seen in the S1 analysis of BE8 RNA at the zero time point (Figure 12) and the presence of bands under the 4 kb transcript upon Northern

analysis (Figure 8) also implies that this RNA is a product of processive degradation of longer transcripts in the 3'-to-5' direction

If the 0 6 kb RNA was a product of RNA processing, an increase in the amount of this RNA would be expected over time, after the cessation of transcription This increase would not be expected to continue indefinitely, since the amount of RNA present is a balance between the rate of formation and the rate of decay Northern analysis of BE8 RNA from samples taken in 5 minute intervals showed an increase in the concentration of the 0 6 kb RNA 30 to 40 minutes after the addition of rifampicin in the presence of Em (data not shown) If the 0 6 kb RNA were a product of transcriptional termination one would not expect to see an increase in concentration of this transcript after transcription had ceased

To further support this conclusion, no band corresponding to transcriptional termination at this sequence is detected in the *lac* operon in *E. coli* However, when transcription and translation were uncoupled in *E. coli* by transcribing the *lacZ* sequence with T7 RNA polymerase, a 240 nucleotide transcript was detected (Chevrier-Miller *et al.*, 1990) It is unlikely that the T7 polymerase is prematurely terminating since the elongation rate of this RNA polymerase is so high that it out runs the translating ribosomes and apparently is unaffected by their absence (Golomb and Chamberlin, 1974) The authors concluded that the 240 nucleotide RNA was a product of RNA processing

The 3' termini I detected upon S1 analysis of BE8 RNA correspond to nucleotides 264-275 of the *lacZ* transcript Ruteshouser and Richardson (1989) have reported that the *lacZ* sequence from nucleotides 222 to 261 have the potential to form an extremely

stable stem-loop with a Gibbs Free Energy of -41 0 Kcal/mol (Figure 26) These authors reported that if translation was blocked in *E. coli*, processed RNAs could be detected, the 3' ends of the most abundant of these mapped to nucleotides 252 to 258 It was concluded that these RNAs were produced by RNase III cleavage at position 258 followed by exonucleolytic trimming The 0 6 kb RNA may therefore be a product of RNase processing by a processive 3'-to-5' exoribonuclease whose procession may be impeded by a strong stem-loop that forms between nucleotides 222 to 261 of the *lacZ* sequence The formation of a stable stem-loop upstream of an RNA processing site has been shown to confer stability to the remainder of the transcript in the processing of the *puf* operon of *Rhodobacter capsulatas* (Klug *et al* ,1987) While the formation of the 3' terminus of the 0 6 kb RNA may occur via similar processes in *E. coli* and *B. subtilis*, the 0 6 transcript is produced in *B. subtilis* independent of translation but is observed in *E. coli* only when translation and transcription are uncoupled (Chevrier-Miller *et al.*,1990)

I have demonstrated that the decay of *ermC'-lacZ* fusion RNAs was not dependent on translation since the same pattern was seen on Northern blots of RNA isolated from strains in which the fusion contained a functional or nonfunctional SD2 site (Figure 8) Furthermore, the 4 kb transcript also remained stable in the absence of translation In *E. coli* the stability of the *lacZ* transcript has been demonstrated as being dependent on the translational efficiency of the RNA (Yarchuk and Dreyfus, 1991; Yarchuk *et.al* , 1992) The dependence of *lacZ* mRNA on translation has been explained by a series of endonucleolytic cleavages in the 5'-to-3' direction which follow the last translating ribosome (Cannistraro *et.al.*, 1986) The model for this type of degradation is shown in

Figure 1A In this model RNase cleavage in or near the ribosome binding site interrupts the flow of ribosomes As the wave of translating ribosomes moves down the mRNA, the mRNA is left unprotected and a processive degradation occurs in the 5'-to-3' direction During active translation the rate at which the RNase can associate with the mRNA decreases Therefore, if the *lacZ* RNA is not translated it should be rapidly degraded in *E. coli*, which is the observed phenomenon (Yarchuk *et al*., 1992) The fact that translation has no effect on the stability of the 0 6 and the 4 kb transcripts in *B. subtilis* indicates that this model for *lacZ* decay does not hold true in *B. subtilis*

The above quoted studies on the effect of translation on *lacZ* decay in *E. coli* showed that the destabilizing effect during impeded translation was dependent on the 5' sequence of the transcripts since RNA from a *lamB-lacZ-lacY-lacA* fusion rapidly degraded in the absence of translation while the decay of RNA from a *malK-lamB-lacZ-lacY-lacA* fusion was not dependent on translation The decay of the *lamB-lacZ-lacY-lacA* RNA was dependent on the presence of activity of the *E. coli* RNase E, while the decay of the *malK-lamB-lacZ-lacY-lacA* was not (Yarchuk *et al.*,1991) Studies on the decay of lacZ mRNA suggested that RNase E must be able to bind the 5' terminus of *lacZ* RNA and then move downstream to a cleavage site in order for decay to initiate The stability of the *ermC'-lacZ* RNAs in *B. subtilis* was dependent on Em-bound ribosomes in the 5' leader region, which served to protect the 5' end This suggests that in both *E. coli* and *B. subtilis* the rate limiting step for the initiation of decay is the availability of the 5' terminus of an RNA to a 5'-binding RNase. The expression of *ermC'* and *ermC'-lacZ* has been examined in *E. coli* (Hardy and Haefeli,1982, Kirsch and Lai,1984) Transformation of *E.*

coli with a plasmid encoding the *ermC'* gene was found to confer Em resistance However, only a small amount of the methylase protein was detected, even in the presence of Em The expression of β-galactosidase was found to be inducible in the presence of Em in *E. coli*, but the concentration of Em necessary for induction was 40 μg/ml (induction in *B. subtilis* requires 0 02 mg/ml of Em) An accurate analysis of *ermC'* induction is problematic, since *E. coli* may be naturally resistant to Em as indicated by the high concentration of Em required for induction.

DNA fragments encoding endonucleolytic cleavage sites were inserted into the *ermC'* coding sequence to determine if the initiation of decay could occur at sites other than the 5' terminus of *ermC'*. Panganiban and Whiteley (1983b) have demonstrated the ability of Bs-RNase III to cleave both the 16S-23S rRNA spacer region and early transcripts of bacteriophage SP82. I cloned the fragment containing the RNase III cleavage site of the *rrnG* spacer region into the *ermC' Hpa*I site and examined the effect that this sequence had on the stability of *ermC'* mRNA

Northern blot analysis of RNA isolated from a strain containing the *ermC'-rrnG-ermC'* construct such that the inserted *rrnG* sequence was transcribed in the same direction as the ribosomal operon showed no cleavage at the inserted *rrnG* sequence (Figure 18A). If the inserted rrnG sequence encoded a Bs-RNase III cleavage site, why did this RNA remain stable? The cleavage of the 16S-23S ribosomal spacer region of 30S transcripts from *E. coli* ribosomal operons requires the formation of a duplex between the 16S-23S spacer sequence and sequences upstream of the 16S rRNA The transcript folds

to form a stem-loop structure in which the "loop" is the entire 16S RNA sequence with RNase III cleavage occurring in the stem portion of this structure (Young and Steitz,1978) *In vitro* experiments by Panganiban and Whitely (1983b), which showed a Bs-RNase III cleavage in the 16S-23S spacer region used an RNA that was identical to the *B. subtilis* rRNA from the 5' end to nucleotide 400 of the 23S rRNA sequence as a substrate If the same structure is required for cleavage in *B. subtilis* as in *E. coli*, then the insertion of the 16S-23S spacer sequence into the *ermC'* coding sequence would not be sufficient for Bs-RNase III cleavage since the necessary structure could not form This would explain the lack of cleavage of the *ermC'-rrnG-ermC'* transcripts of BE273

A transcript longer than the expected 1,430 nucleotide RNA was detected by Northern analysis of RNA isolated from strain BE273 (Figure 18A) Transcription beyond the *ermC'* transcriptional terminator is not normally detected (Figure 5), indicating that this is an efficient terminator Why then does insertion of the *rrnG* spacer region in the same orientation as the ribosomal operon produce this read-through? Antitermination signals are known to exist in ribosomal operons to aid in the transcription of such long RNAs (Morgan,1980). The existence of antitermination sequences in the spacer region of *B. subtilis* ribosomal operons has been reported (Berg *et al.*,1989) The antitermination signal of ribosomal operons consists of three consensus sequences termed Box B, Box A and Box C The importance of Box C for antitermination is questionable since it is not present in all antiterminators and mutations in this sequence have no effect on antitermination. Mutations in Box B and Box A have an effect on antitermination, demonstrating that these sequences play a role The inserted fragment of the *rrnG* spacer

region encoded a Box B and a Box A sequence A deletion analysis of the inserted *rrnG* sequence has been done (Bechhofer, unpublished) Deletions in which the Box A sequence was no longer present resulted in a loss of antitermination This indicates that the antitermination seen in transcripts of BE273 was caused by the presence of Box A

Northern blot analysis of RNA from strain BE274, which contained the *rrnG* sequence transcribed as the reverse complement of the ribosomal operon, is shown in Figure 18B RNAs transcribed from this fusion were not inducibly stable in the presence of Em-bound ribosomes Insertion of the fragment encoding this sequence into different locations produced transcripts that were also not stabilized by Em-bound ribosomes This result indicated that the inserted sequence was sufficient for the loss of stability and that the destabilizing effect was not the result of formation of alternative secondary structures between the RNA transcribed from this inserted sequence and neighboring sequences The reverse complement sequence of the spacer region for the *rrnG* operon was subjected to folding by computer modeling (Devereux *et al.*, 1984) and no reasonable Bs-RNase III cleavage site could be found, indicating that this sequence did not contain a Bs-RNase III cleavage site

If the sequence transcribed as the reverse complement of the *rrnG* spacer region did not encode a Bs-RNase III cleavage site, why were transcripts containing this sequence unstable? It is possible that this sequence acts as a target site for some other endoribonuclease Alternatively, since transcription of this sequence produces an RNA sequence that is complimentary to the transcribed sequence of the ribosomal operon spacer region, cleavage may occur in RNA duplexes which form between these two

transcripts Although the mechanism for destabilization by this sequence remains unknown, one can conclude that an alternate site for the initiation of decay can occur and that the accessibility of the 5' terminus of the *ermC'* transcript is not essential for the initiation of decay

Northern blot analysis of BE274 RNA demonstrated that antitermination also occurred when the inverted *rrnG* sequence was inserted into *ermC'* (Figure 18B) The degree of antitermination was higher in this strain than in BE273 The sequence transcribed as the reverse complement of the ribosomal transcript does not encode the Box A antitermination sequence What then is the mechanism by which antitermination occurs in this construct? It has been shown in the λ system that antitermination occurs when the Box A sequence was not transcribed as part of the antiterminated RNA, but was present on an RNA that formed a duplex with the 5' end of the antiterminated RNA (A Das, unpublished) It has been proposed that the presence of the Box A sequence in the vicinity of the elongation complex, without Box A actually being transcribed, is sufficient to cause antitermination A model has been proposed in which antitermination occurs when duplexes form between the RNA being transcribed and a complimentary RNA that contains a Box A sequence (Figure 27, Squires,C L and Squires C , personal communications) If this model is true then I would propose that both the antitermination effect and the loss of stability of mRNAs encoding the reverse complement of the *rrnG* spacer region are due to the ability of this sequence to form duplexes with the spacer region of ribosomal RNA

The results of the insertion of the ribosomal spacer region were interesting but they did not give conclusive evidence that an internal cleavage site could act as the target for the initiation of decay in *B. subtilis*. To examine this, it was necessary to insert a defined Bs-RNase III cleavage site into the *ermC'* coding sequence. As was mentioned above, the SP82 terminal repeat produces early transcripts which are targets for Bs-RNase III. One of these target sites was amplified by PCR and cloned into the *BclI* site of the sequence encoding the *ermC'* methylase. Strain BE301 contains the inserted sequence so that it is transcribed in the same orientation as in SP82. Northern blot analysis of RNA isolated from this strain demonstrated that this RNA was rapidly processed and that the full length transcript was barely detectable (Figure 22). Mapping of the 3' terminus of the upstream cleavage product and the 5' terminus of the downstream cleavage product demonstrated that the cleavage of the full-length transcript occurred between nucleotides 215 and 216 of the inserted sequence(thick arrow, Fig. 23B). Computer modeling of the sequence surrounding this site produced the same stem-bulge-stem-loop as proposed by Panganiban and Whiteley (1983a; Figure 23B) However, the site of cleavage in BE301 does not correspond with that reported from both *in vivo* and *in vitro* experiments (Figure 23B , small arrow). Recent experiments using a partially purified *B. subtilis* extract have demonstrated cleavage at the same site *in vitro* as the one I mapped *in vivo* (Mitra and Bechhofer, unpublished). The mapped cleavage site in BE301 RNA is similar to the cleavage site of three of the five RNase III cleavage sites in T7 early RNA (Dunn and Studier, 1983). These sites of cleavage occur between AU and G residues of an internal

bulge sequence that contains four unpaired nucleotides forming the 5' side of the bulge and five unpaired nucleotides forming the 3' side of the bulge (Figure 23B)

The results of Northern blot analysis of RNA isolated from strain BE303, which contained the inverted SP82 Bs-RNase III sequence, demonstrated that while the reverse complement of this sequence was also cleaved, it was not processed as readily as the sequence transcribed in the orientation as SP82 (Figure 22) This is evident from the fact that full length RNA is readily detectable in this strain The 3' terminus of the upstream cleavage product of BE303 was mapped and this was determined to be at position 134 of the SP82 sequence (numbers given are for same orientation as SP82, not inverted sequence) Although a similar structure can form in BE303 RNA as in BE301 RNA, cleavage does not occur in the same site as in BE301

The patterns of decay for the cleavage products of BE301 and BE303 are quite different The upstream cleavage product of BE301 has the 5' leader region of *ermC'* as its 5' proximal sequence and the first 216 nucleotides of the SP82 Bs-RNase III sequence as its 3' proximal sequence This RNA was not stabilized by the presence of Em-bound ribosome stalling in the leader region (Figure 22A) This indicated that after cleavage the 3' terminus of this transcript was accessible to 3'-to-5' exonucleases that rapidly degraded the upstream cleavage product The degradative event that ensued after the initial endonucleolytic cleavage of the full length transcript was not dependent on the accessibility of the 5' terminal sequence Why was this 3' terminus a target for RNase attack while other 3' termini are protected when a ribosome is stalled in the *ermC'* leader region? Most likely, the 3' proximal region does not form a protective secondary structure

after cleavage and is therefore accessible to 3'-to-5' exoribonucleases. This would explain the inability of Em-bound ribosomes to protect this transcript while being able to protect the diverse downstream sequences in the fusion experiments (Figure 11). The 3' termini of the fusions should all terminate in a stable secondary structure since they are all formed by transcriptional termination. In any event, the decay of the upstream cleavage product demonstrated that the initiation of mRNA decay in *B. subtilis* can initiate at sites other than the 5' proximal region.

The downstream cleavage product of BE301 was stable despite the absence of Em-bound ribosomes. The 5' proximal sequence of this RNA was subjected to computer modeling and it was found that the 5' terminus can be sequestered into a stem-loop (Figure 23C). This stem-loop is rather weak (-4.0 kcal/mol) and is probably not the cause of this stability. Protection may occur by Bs-RNase III itself, which may remain bound to the 5' proximal sequence after cleavage. Deletion analysis demonstrated that cleavage still occurs if SP82 sequences up to the *Alu*I site at nucleotide 227 are present, but the downstream cleavage product does not remain stable in this case (Hue and Bechhofer, unpublished). This evidence argues against the possibility that protection is provided by the endoribonuclease remaining bound to the RNA since the deletion of sequences downstream of 227 yielded an RNA which was cleaved and did not remain stable. In order for both cleavage and stability to occur, sequences up to the *Hpa*I site at position 255 are required. Although a ribosome binding site (RBS) is present 10 nucleotides upstream of the *Hpa*I site (Figure 21), deletion of SP82 sequences downstream of the *Hpa*I site that left no initiation codon near its RBS still resulted in a stable downstream cleavage product,

showing that ribosome binding does not play a role in stability of the downstream cleavage product. Another explanation for the stability of the downstream cleavage product of BE301 RNA is that a protein may bind the 5' end of this RNA and act as a *trans*-stabilizer. The presence of such a protein could be tested for by RNase Footprinting analysis or by gel-shift assays.

The discovery that the downstream sequence of BE301 remained stable was almost ironic. In the process of using an inserted endonucleolytic cleavage site to examine alternate sites for the initiation of decay, we discovered another 5' stabilizing structure. Future experiments may be performed in which the SP82 sequence that encodes the 5' nucleotides of the downstream cleavage product are transcribed as the first nucleotides of an mRNA. This would determine whether stability is dependent on the formation of the 5' terminus by cleavage or if the presence of the sequence at the 5' terminus confers stability by itself.

The upstream cleavage product of BE303 contains the leader region of *ermC'* as its 5' proximal sequence and the reverse complement of the SP82 sequence from nucleotides 370 to 134. This RNA was found to be inducibly stable in the presence of Em-bound ribosomes (Figure 22C). The protection of the 5' terminal region of this RNA was essential for its stability as is demonstrated by its rapid decay in the absence of Em. The 3' terminal sequence was not a target of RNase attack and this may be due to some secondary structure that forms in this region. Computer modeling of potential secondary structure formed by the 3' proximal sequences has been done. The 3' sequences of this RNA can form a stable stem-loop structure (-19.5 kcal/mol; Figure 23D). Comparing the

stability of this RNA with the upstream cleavage product of BE301 indicates that the instability of the upstream BE301 RNA is not an inevitable result of endonucleolytic cleavage, but rather depends on the nature of the 3' ends of the RNA.

The downstream cleavage product of BE303 has the reverse complement sequence of the SP82 Bs-RNase III sequence from nucleotides 135 to 1 as its 5' proximal sequence. The 3' proximal sequence is that of the *ermC* transcript. This transcript was found to be unstable in the presence and absence of Em. The instability of this transcript was probably due to the accessibility of its 5' proximal region to RNases. This may be due to the lack of any secondary structure at the 5' end of the RNA that would protect RNA from decay

Figure 26 Possible stem-loop structure of the 3'-terminal sequence of the 0.6 kb RNA The numbers are for *lacZ* sequence (Ruteshouser and Richardson, 1989). The free energy is given

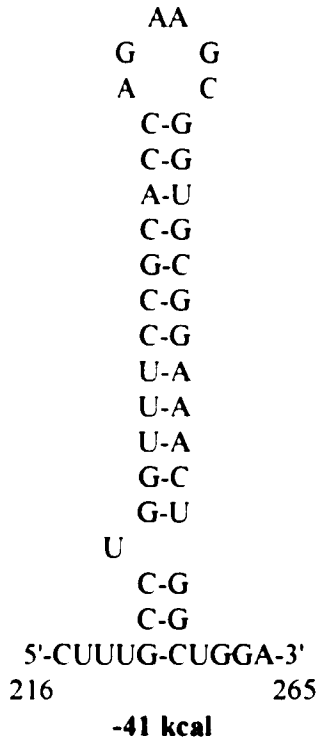
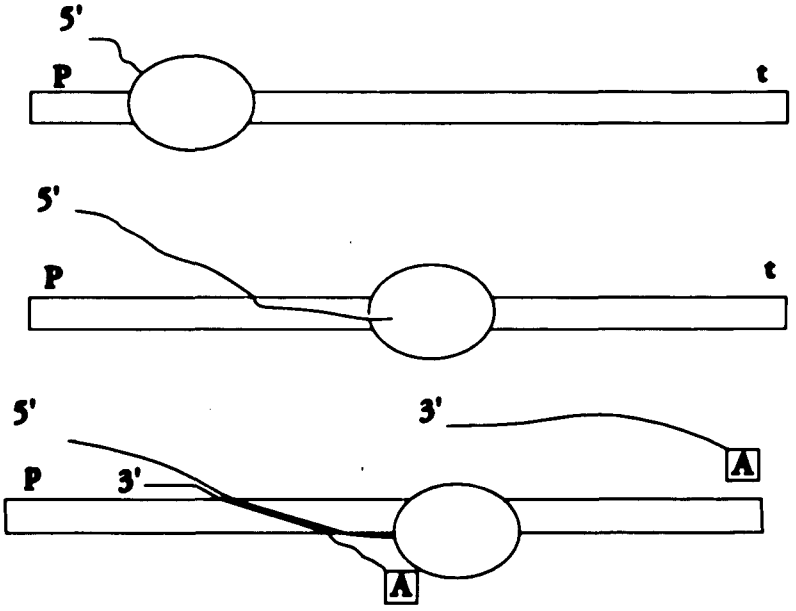

AA
G G
A C
C-G
C-G
A-U
C-G
G-C
C-G
C-G
U-A
U-A
U-A
G-C
G-U
U
C-G
C-G
5'-CUUUG-CUGGA-3'
216 265
-41 kcal

Figure 27 Model for antiitermination by a non-transcribed Box A sequence (Squires and Squires, personal communications) The RNA polymerase complex (the oval) binds the promoter and transcription begins As the elongation process continues, an RNA sequence is transcribed that is complementary to an RNA that contains a Box A sequence (box) An RNA duplex forms and the presence of the Box A sequence on the non-transcribed causes antitermination



Antitermination

Conclusion:

Currently there is a dearth of understanding of the mechanisms by which mRNA decay initiates Mechanisms for the initiation of decay of specific mRNAs have been reported, but a general pathway has not been described for this process The majority of the literature on mRNA decay describes research done in *E. coli* Recent reports indicate that the endoribonuclease RNase E is central to the initiation of decay

RNase E has been described as a 5'-binding endonuclease that is involved in the initiation of mRNA decay in *E. coli* (Bouvet and Belasco,1992) The inactivation of this enzyme led to an increase in the level of *E.coli* mRNAs, demonstrating that RNase E plays a role in the initiation of decay of *E. coli* mRNAs in general (Ono and Kuwano,1979) The initiation of decay by this enzyme requires an accessible 5' terminus and secondary structures that sequester this terminus were found to increase stability Structures that block the binding of this enzyme confer stability to the mRNA even if downstream processing sites are present (Emory and Belasco,1992) This data indicates that the initiation of mRNA decay requires an accessible 5' terminus even though the RNase target sites may be situated downstream in the body of the message A model for this type of decay is shown in Figure 1B

My results indicate that the initiation of mRNA decay in *B. subtilis* is also dependent on an accessible 5' terminus The 5' leader region of *ermC* has been shown to act *in cis* to protect diverse and lengthy sequences It is doubtful that all these sequences lack RNase target sites Rather, the presence of a 5' stabilizing sequence

makes internal RNase target sites inaccessible. This implies that the initiation of decay occurs by some yet unidentified ribonuclease(s) binding in the 5' proximal region of an mRNA. This 5'-binding ribonuclease may then migrate along the transcript or "loop" to access downstream cleavage sites.

If this model were true, then the initiation of mRNA decay in both *E. coli* and *B. subtilis* occurs through 5' proximal inspection. The actual decay of the mRNAs does not have to proceed in the 5'-to-3' direction, but the mRNAs would be inspected in this direction. Inspection of mRNA in the 5'-to-3' direction would be consistent with the directionality of other molecular events such as transcription, translation and DNA replication, which all occur in the 5'-to-3' direction. The hypothesis that only the inspection and not the decay of mRNAs occurs in this direction is consistent with the observations that various mRNAs decay with different directionality.

The results presented in this thesis have led to several conclusions. The model proposed by Kennell (Figure 1A) for the initiation of mRNA decay in the 5'-to-3' direction in *E. coli* is not applicable to *B. subtilis*, since the pattern of mRNA decay is the same for translated and untranslated mRNAs. Therefore, the initiation of decay cannot be dependent on the ribosome density on the mRNA. Inspection of the 5' terminal region of an mRNA appears to be the rate limiting step for the initiation of mRNA decay in *B. subtilis* in general as is suggested by the ability of the *ermC* leader region to confer stability to diverse downstream sequences of *Bacillus* mRNAs in the presence of Em-bound ribosome. Although inspection at the 5' terminus of an mRNA appears to be the general mechanism for the initiation of mRNA decay in *B. subtilis*, alternate modes

exist for the initiation of decay The inability of a stalled ribosome at the 5' terminal region of an RNA to confer resistance to the RNA when a downstream endonucleolytic cleavage site is present demonstrates that the initiation of decay can occur without 5' inspection I also conclude that stable cleavage products can remain after the initial cleavage event, demonstrating that the decay process can be blocked and can lead to differential stability of segments of mRNA in *B. subtilis* This is demonstrated by the fates of the upstream and downstream cleavage products of the RNA fusions of strains BE301 and BE303

This work has been far from definitive Only a handful of RNAs have been examined and even in this small sampling exceptions have been found I have described transcripts that are not stabilized by 5' proximal protection This indicates that alternate pathways of decay can occur which are not dependent on the availability of the 5' proximal region of an mRNA To truly understand the process of mRNA decay, the RNases involved in mRNA decay need to be isolated and defined In this area *B. subtilis* research is lagging behind that of *E. coli* Once these RNases have been defined the effect of their inactivation on mRNA decay must be assessed to understand what role they play in the degradative process

Appendices

Appendix A

Table II

Table of Strains and Plasmids

Strain	Plasmid	Description
BG1	None	BD170, *trpC2, thr-5*
BG2	None	IS75, *his, leu, metB5*
BG11	None	BD224, *trpC2, thr-5, recE4*
BE4	pBD142	Contains *ermC* and *cat*
BE8	pBD246	Contains *ermC-lacZ* fusion
BE168	pSD127	Contains *ermC-penP* fusion
BE223	pSD162	*Cla*I deletion of pBD246 with *cat* in opposite orientation as pBD246
BE224	pSD163	*Cla*I deletion of pBD246 with *cat* in same orientation as pBD246
BE253	pSD191	Replacement of *ermC BclI-Hpa*I with *lacZ BamHI-Hpa*I
BE273	pSD210	Insert of *rrnG* spacer into *Hpa*I of *ermC*, same orientation as *rrnG*
BE274	pSD211	Insert of *rrnG* into *Hpa*I of *ermC*, opposite orientation as *rrnG*
BE275	pSD212	Contains *ermC* terminator deletion
BE276	pSD213	SD2 mutant of pSD210

Strain	Plasmid	Description
BE277	pSD214	SD2 mutant of pSD211
BE278	pSD215	Contains *ermC'-sacA* fusion
BE279	pSD216	Insert of ribosomal spacer region into *ermC' BclI* site same orientation as *rrnG*
BE280	pSD217	Insert of ribosomal spacer region into *ermC' BclI* site opposite orientation as *rrnG*
BE285	pSD222	Contains *ermC'-sacB* fusion
BE290	pSD227	Insert of ribosomal spacer regon into *BclI* site of *ermC'-lacZ*, same orientation as *rrnG*
BE291	pSD228	Insert of ribosomal spacer region into *BclI* of *ermC'-lacZ*, opposite orientation as *rrnG*
BE292	pSD229	Duplication of *lacZ PvuII-HpaI* fragment in *ermC'*
BE301	pSD238	Insert of SP82 sequence into *ermC'*, same orientation as SP82
BE303	pSD240	Insert of SP82 sequence into *ermC'*, opposite orientation as SP82
BE314	pSD251	Contains *ermC'-epr* fusion

Appendix B

Isolation of RNase Mutants

I attempted to isolate *B. subtilis* strains which expressed temperature sensitive mutants of RNases. Our original strategy was to insert a single copy of the *ermC'* gene into the *B. subtilis* chromosome, mutagenize the cells and look for colonies with an increased resistance to Em. An increase in resistance to Em could be due to a decrease in the rate of decay of the *ermC'* mRNA, which would indicate that an RNase involved in the decay of the ermC mRNA was affected. The *ermC'* gene was cloned into plasmid pBD385, which contained resistance genes for kanamycin and phleomycin and a temperature sensitive replicon, yielding plasmid pSD123. Plasmid pSD123 DNA was used to transform BG11 cells which are *trpC2, thr-5, recE4*. This strain was denoted BE158. BE158 cells were grown up overnight in VY media containing 1 μg/ml of phleomycin at 32° C. 5 ml of the overnight culture was added to 175 ml of fresh VY containing phleomycin and was grown at 50° C with shaking to select for cells in which the plasmid had integrated. The cells were grown to stationary phase and 5 ml of the culture was used to inoculate 95 ml of VY containing phleomycin. The cells were then grown to stationary phase at 50° C. 0.1 ml of the culture was then spread on TBAB plates containing phleomycin and incubated at 50°C overnight.

Isolated colonies were selected from these plates and were tested for the presence of plasmid DNA by visualization of cellular DNA on an agarose gel and the ability of the DNA to confer Kanamycin resistance to BG1 cells transformed with the DNA. Cells that did not contain DNA were selected and chromosomal DNA from these cells was digested

with *Bam*HI and *Bcl*I and subjected to Southern blot analysis using an *erm(*' probe Strain BE174 contained an integrated copy of the plasmid and was used for mutagenesis

BE174 cells were grown overnight in VY media 10 ml of the culture was taken and spun at 5,000 rpm for 3 minutes The cells were resuspended in 5 ml of 1X Spizizen salts (SS), centrifuged as above, and the process repeated The cells were then resuspended in 2 5 ml of 1X SS 0 5 ml of cells were added to 5 ml of 1X SS with or without 0 2 ml of Ethylmethylsulfonate (EMS) The cells were incubated at 37° C with shaking and 1 ml samples were taken at 0, 10 and 20 minute time points and placed into eppendorf tubes The samples were washed twice with 1X SS and resuspended in 1 ml of 1X SS 0 1 ml was taken and diluted 10^{-4}, 10^{-5} and 10^{-6} and 0 1 ml was plated onto TBAB plates and incubated overnight at 37° C The percentage killing was determined by comparing the number of colonies that grew on plates after EMS treatment to the number of untreated cells that grew 0 5 ml of the mutagenized cells were added to 4 5 ml of VY and grown overnight at 37° C 0 1 ml of cells grown overnight in VY, as well as 10^{-1} and 10^{-2} dilutions were plated onto TBAB plates containing 5 µg/ml of rifampicin and incubated overnight The remainder of the cells grown in VY were frozen by adding an equal volume of 50% glycerol and freezing at -70° C

The percent mutagenesis was determined by comparing the number of rifampicin resistant colonies from treated cells to the number from untreated cells The 20 minute treatment with EMS gave the highest ratio of percent mutagenesis to percent killing

Frozen stocks of the cells treated for 20 minutes with EMS were diluted 10^{-2}, 10^{-3}, and 10^{-4} in VY and 0 2 ml was plated onto TBAB plates containing 0 5, 1 0 or 2 0

μg/ml of Em and 5 μg/ml of kanamycin. The plates were incubated overnight at 37°C and colonies were selected that demonstrated an increased resistance to Em compared to untreated cells. DNA from these cells was then subjected to Southern analysis to test if the increase in resistance was due to a possible rearrangement of the inserted sequence or a mutation of *ermC*. If the Southern blot of the strain expressing an increase in Em resistance was the same as the Southern of the strain prior to mutagenesis, this would indicate that the increase in Em resistance was due to an RNase mutant. It was found, however, that the increase in Em resistance was produced by multiplication of the number of copies of the *ermC* gene integrated into the chromosome.

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