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Changes in the stability of specific mRNA species in response to growth stage in *Bacillus subtilis*

(succinate dehydrogenase/aprE/cell differentiation/posttranscriptional control/mRNA decay)

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ABSTRACT **In this study we compared the cellular concentrations and stability of the mRNA transcribed from the *aprE* (subtilisin) gene (a gene preferentially expressed in stationary growth phase) with those of a vegetative mRNA, succinate dehydrogenase (SDH) mRNA. The subtilisin transcript was shown to be at least 3 times more stable in early stationary phase than it is 2 hr further into stationary phase. When cells were shifted from maximum expression of the subtilisin transcript in stationary phase to physiological conditions, which allowed for the resumption of vegetative growth, the cellular concentration of the subtilisin mRNA decreased rapidly. We conclude that mRNA degradation is one of the means by which the cellular concentrations of the SDH and subtilisin transcripts are adjusted in response to growth stage.**

The study of gene expression has primarily been involved in understanding the mechanisms of transcription and translation. One important element that has been relatively ignored is the mechanism of mRNA stability. There is a great deal of variability in the stability of different messages, which can vary in *Escherichia coli* from seconds to a fraction of an hour (1–3) and in eukaryotes from a few minutes to days (4, 5). In both prokaryotes and eukaryotes the half-lives of certain mRNA species have been shown to be altered in response to growth (3, 6–8). The targets in the RNA and the enzymes and/or factors responsible for the different decay rates of mRNA are now the subject of investigation in both prokaryotic and eukaryotic systems (for reviews, see refs. 9 and 10).

Bacillus subtilis has been used as a model system to study developmental gene expression (for a review, see ref. 11). *B. subtilis* grow exponentially in a nutrient medium. When the environment is no longer favorable for growth (if, for example, there is a limitation of nutrients), they cease to grow exponentially. In stationary phase the cell undergoes a series of morphological changes that eventually lead to the creation of a spore (11). The control of gene expression at the level of transcriptional regulation has been extensively investigated in *B. subtilis* (for a review, see ref. 12). In this report we have examined whether, in addition to transcriptional regulation, RNA degradation contributes to the modulation of gene expression in this organism.

For this analysis we have chosen to use two transcripts encoding the enzymes succinate dehydrogenase (SDH) and AprE (subtilisin). SDH is a membrane-bound enzyme in the Krebs cycle that oxidizes succinate to fumarate. Subtilisin is a secreted alkaline protease. The SDH mRNA is preferentially expressed during vegetative growth (6, 13), while the subtilisin mRNA is preferentially expressed during stationary phase and is used as an indicator of sporulation-specific transcription (14, 15). A schematic drawing of the mRNAs for both enzymes is shown in Fig. 1.

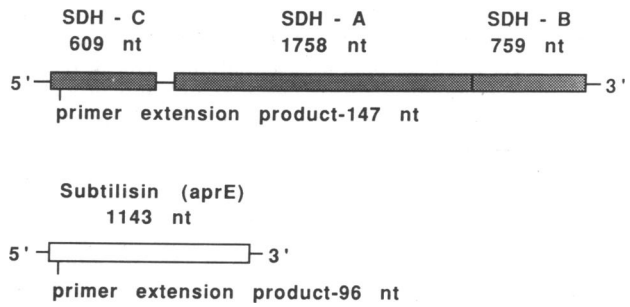

FIG. 1. Schematic diagrams of the SDH operon and the subtilisin transcript. The SDH mRNA is a polycistronic transcript that contains the structural genes for the three subunits of SDH: cytochrome b558 (sdhC), a flavoprotein (sdhA), and an iron-sulphur protein (sdhB) (13). The subtilisin mRNA is a monocistronic transcript. The protein is translated as a propolypeptide and contains a signal sequence (16). The coding regions of both transcripts are shown as boxes and the noncoding regions as thin lines. The 5′ and 3′ ends of the transcripts are indicated; the 5′ end of both transcripts has been mapped, while the exact position of the 3′ end of both transcripts is unknown. The names and lengths [in nucleotides (nt)] of the coding regions are indicated. The locations of the primers used for the primer extension analyses and the lengths in nt of the extension products are shown; the sequence of the primers is included in *Materials and Methods*.

We used the subtilisin and SDH transcripts to compare the cellular concentrations and stability of a stationary phase-specific transcript with those of a vegetative-specific transcript. We found that RNA degradation is one of the means by which the cellular concentration of these transcripts is adjusted and that the degradation is specific. At a given growth stage, the stability of the two transcripts is different. In addition, a change in physiological conditions permitting the resumption of vegetative growth from stationary phase rapidly affects the cellular concentration of the subtilisin mRNA.

MATERIALS AND METHODS

Bacteria and Medium. The bacterial strain used was *B. subtilis* 3G18 (*ade met trpC2*). Liquid cultures were grown in nutrient sporulation medium (NSPM) (17). In Fig. 4, 100 μg of rifampicin was added per ml (ref. 18; unpublished data). For the dilution studies (see Figs. 5 and 6) 25 ml of a T_2 culture was diluted into 100 ml of prewarmed NSPM; a T_2 culture is a culture 2 hr after the start of the stationary growth phase at T_0.

Preparation of RNA. RNA was extracted as described (6) except as follows: after the lysozyme treatment, an equal

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Abbreviations: SDH, succinate dehydrogenase; T_0, T_1, T_2, T_3, and T_4, time in hours (subscript) after the start of the stationary phase of growth at T_0.

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volume of a buffer containing 0.1 M NaOAc (pH 4.0), 2% sodium dodecyl sulfate (SDS), 25 mM EDTA, 5 mM 1,10-phenanthroline, and 4 mg of heparin per ml was added. The cells were lysed as described above. Lysates were treated with proteinase K (2 mg/ml; Boehringer Mannheim) at 37°C for 20 min, extracted with 1:1 (vol/vol) phenol/chloroform, and precipitated with ethanol. The pellets were treated with 30 units of RNase-free DNase (Cooper Biomedical) in TKM buffer (25 mM KCl/2.5 mM $MgCl_2$/50 mM Tris, pH 6.7) for 30 min at room temperature. The samples were extracted with phenol/chloroform and precipitated with ethanol. The RNA concentrations were determined at 260 nm with a Perkin–Elmer 550 SE spectrophotometer.

Analysis of RNA. RNA blot-hybridization analysis (Northern analysis) was performed as described (19) with 10 μg of RNA per sample. RNA markers (Bethesda Research Laboratories) were used for size determinations. The RNA was blotted onto Hybond-N filters (Amersham) and processed according to the manufacturer's instructions. Primer extension reactions were performed as described (20) with 10 μg of RNA per sample and avian myeloblastosis virus reverse transcriptase [according to the manufacturer's (Bioexcellence) instructions, 2 units per sample] except that the RNA and primer were denatured by heating to 80°C for 5 min and were hybridized by cooling on ice. Primers were end-labeled as described (19) by using phage T4 polynucleotide kinase (Pharmacia). The primers used as probes for the Northern blots and primer extension reactions were 24-mers and were designed to be in the coding regions of both transcripts (see Fig. 1): SDH, 5′-GTA TGA CGC CAA GCA AGG AAT GCA-3′; and subtilisin, 5′-CGC AAA CAA CAA GCT GAT CCA CAA-3′. The extension products were run on 7 M urea/1× TBE/6% polyacrylamide gels (19:1 acrylamide/methylenebisacrylamide; 1× TBE = 0.09 M Tris/0.09 M boric acid/2.5 mM EDTA, pH 8.3). The gels were exposed to Fuji RX x-ray film. For half-life determinations, at least two separate RNA preparations were analyzed and two exposures of each film were scanned with a Helena Laboratories Data Center.

RESULTS

To determine the cellular concentrations of the SDH and subtilisin transcripts during vegetative growth and stationary phase, we used RNA that had been extracted from samples of cells taken at different times across the growth curve and analyzed them by primer extension. The growth curve and the points at which the samples were taken are shown in Fig. 2*D*. The primers used for the primer extension reactions were designed to be in the 5′ coding regions of both genes, and the lengths of the expected extension products are shown in Fig. 1. The cellular concentration of the SDH mRNA was highest during vegetative growth (Fig. 2*A*, lanes 1 and 2) and decreased as the cells reached stationary phase (Fig. 2*A*, lanes 3–6). In contrast, the cellular concentration of the subtilisin mRNA increased as the cells approached stationary phase (Fig. 2*B*, lanes 3 and 4) and decreased about 2 hr later (Fig. 2*B*, lanes 5 and 6). The same RNA samples were used to analyze both of these transcripts. The unique extension products that we observed confirmed the reported transcription start sites for the SDH (13) and subtilisin (14, 15) transcripts. To quantitate the cellular concentrations of the SDH and subtilisin mRNAs, the gels shown in Fig. 2 *A* and *B* were scanned with a densitometer (Fig. 2*C*). The level of each transcript is normalized to its maximum level. Thus, we do not mean to compare the relative amounts of the SDH and subtilisin transcripts. No adjustment has been made for the expected cellular decline of ribosomal RNA as the bacterial growth rate slows (3).

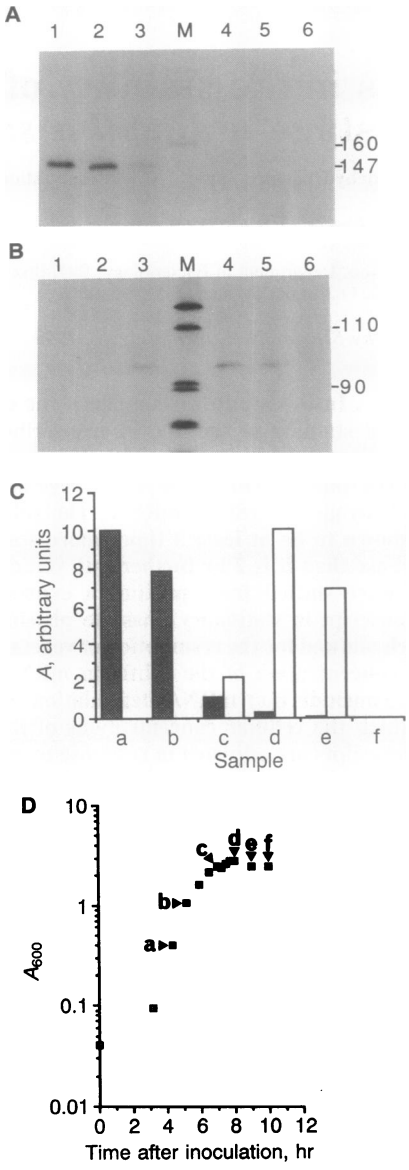

FIG. 2. Cellular concentrations of the SDH and subtilisin transcripts during growth of *B. subtilis*. (*A*) Primer extension analysis of the SDH mRNA. (*B*) Primer extension analysis of the subtilisin mRNA. Lanes: 1–6, Samples a–f in *D*, which correspond to mid-logarithmic, late logarithmic, T_1, T_2, T_3, and T_4 growth periods, respectively (T_0 is defined as the start of the stationary phase, and the subscripts refer to the time in hr after T_0); M, end-labeled pBR322 digested with *Hpa* II. (*C*) Plot of the densitometric analysis of the gels shown in *A* and *B*. The filled bars are the SDH transcript and the open bars are the subtilisin transcript. (*D*) Growth curve and points at which samples were taken for RNA analysis (shown by triangles and letters).

Since the primers that we had chosen for the primer extension analysis were designed to analyze the 5′ ends of both transcripts, we wanted to confirm that the results that we observed reflected the fate of the entire transcripts. For this purpose we analyzed the RNA samples by Northern analysis, using the primers from the primer extension analysis as probes. As can be seen in Fig. 3*A*, the SDH probe hybridized with a transcript of about 3.5 kb (see the schematic drawing in Fig. 1). In addition, the expression of the full-length transcript was highest during vegetative growth (compare lanes 1 and 2 in Fig. 3*A*) as was shown in the primer extension analysis (Fig. 2*A*). The subtilisin probe hybridized with a transcript of the expected length of about 1.4 kb (see

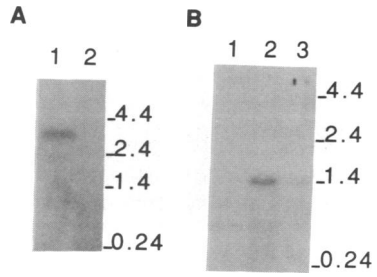

FIG. 3. Cellular concentrations of the SDH and subtilisin transcripts by Northern analysis. (*A*) SDH transcript. Lanes: 1, logarithmic growth; 2, growth at T_4. (*B*) Subtilisin transcript. Lanes: 1, logarithmic growth; 2, growth at T_2; 3, growth at T_4. The probes for the analysis were the primers used in Fig. 2. RNA markers were used for size determinations.

the schematic drawing in Fig. 1 and see Fig. 3*B*). The expression of the full-length transcript was highest during stationary phase (compare lanes 1, 2, and 3 in Fig. 3*B*) as was shown in the primer extension analysis (Fig. 2*B*). We saw no evidence of accumulated cleavage products with either transcript as their respective cellular concentrations decreased. However, the probes used would only have detected those cleavage products with intact 5'-ends.

The decrease in the cellular concentration of the SDH transcript as cells reach stationary phase has been shown to correlate with a corresponding decrease in the half-life of the transcript (6). The half-life of the SDH transcript was shown to be 2.6 min during vegetative growth and decreased to 0.6 min at T_1 (21). A similar destabilization of mRNA half-life as cells approach stationary phase has been found for a second Krebs cycle enzyme; 2-oxoglutarate dehydrogenase (L. Melin, O.R., L. Hederstedt, A.v.G., P. Carlsson, unpublished data). These results could suggest a general destabilization of mRNA stability as cells reach stationary phase. Therefore, we analyzed the half-life of the subtilisin mRNA over the time that it is expressed. Rifampicin (100 μg/ml) was added to the cultures at a time in the growth curve corresponding to samples c (at T_1) and e (at T_3) in Fig. 2*D*, and samples were collected for RNA analysis over a 30-min time period. The primer extension reactions are shown in Fig. 4*A* (T_1) and *B* (T_3), and a densitometric analysis of these gels is shown in Fig. 4*C*. We found that less than 40% of the subtilisin message was degraded after 30 min, and by extrapolation the half-life of the subtilisin mRNA was about 40 min at T_1. The half-life of the subtilisin transcript decreased to 12 ± 0.9 min at T_3, the time at which the steady-state level of the transcript decreased. At both T_1 and T_3, the subtilisin transcript exhibited a linear pattern of decay. The results show that the subtilisin transcript is extremely stable as the cells approach stationary phase (T_1) and are consistent with the suggestion that there is not a destabilization of all mRNA species in the transition from vegetative growth to stationary phase.

These results, in conjunction with the results on SDH and 2-oxoglutarate dehydrogenase, suggest that vegetative transcripts are selectively degraded as cells approach stationary phase. We wondered whether stationary-phase transcripts would be selectively degraded in vegetative-phase cells. For this purpose we grew a culture of *B. subtilis* to T_2 and then diluted a portion of the culture back into fresh warmed medium where the cells would be expected to grow exponentially. Samples of cells were taken for RNA analysis, and the RNA was analyzed for the cellular concentrations of the SDH (Fig. 5*A*) and subtilisin (Fig. 5*B*) transcripts. Lanes 1–4 in Fig. 5 *A* and *B* confirm the results shown in Fig. 2 *A* and *B*. Lanes 5–8 of Fig. 5 *A* and *B* show the steady-state levels of the SDH and subtilisin transcripts immediately after dilution of the cells and at 30-min intervals thereafter. The SDH transcript (which was not highly expressed at T_2; Fig. 5, lanes 4) did not accumulate immediately after the dilution and began to accumulate somewhere between 30 and 60 min after the dilution (Fig. 5*A*, lane 7). In contrast, the cellular concentration of the subtilisin transcript decreased immediately after the dilution (compare lanes 4 and 5 in Fig. 5*B*). The lack of expression shown in the 30-min sample (Fig. 5*B*, lane 6) persisted until at least 90 min after the dilution (Fig. 5*B*, lanes 7 and 8). The level of the SDH transcript equaled and exceeded that shown in lanes 1 and 2 of Fig. 5*A* by 60 min after the dilution (Fig. 5*A*, lane 7). This is probably because the samples used in lanes 1 and 2 were not taken at the maximal expression of the SDH mRNA in vegetative growth. The results shown in Fig. 5 *A* and *B* were analyzed with the same RNA samples. The cell growth was followed prior to and subsequent to the dilution (Fig. 5*C*); the cells began to grow logarithmically about 15 min after the dilution and continued to do so for the remainder of the time over which samples were collected.

We postulated that the decrease in the cellular concentration of the subtilisin mRNA immediately after dilution of the cells could be due to a rapid destabilization of the transcript. Therefore, we analyzed the disappearance of the subtilisin transcript after dilution of the culture. The results of the primer extension reactions are shown in Fig. 6*A*. A densitometric analysis of the gel is shown in Fig. 6*B*, and the points in the growth curve at which samples were taken for RNA analysis are shown in Fig. 6*C*. Inspection of Fig. 6*B* shows that, within 5 min after dilution of the culture, 50% of the subtilisin transcript disappeared. In this experiment transcription was not stopped by the addition of rifampicin (as it was in Fig. 4), but the data indicate rapid cessation of transcription of the *aptE* transcript after dilution of the culture.

DISCUSSION

In this study we compared the cellular concentrations and stability of the mRNA transcribed from the subtilisin gene (a gene preferentially expressed in stationary phase) with those of a vegetative mRNA, SDH mRNA. We found that the stability of the subtilisin mRNA is growth-phase regulated.

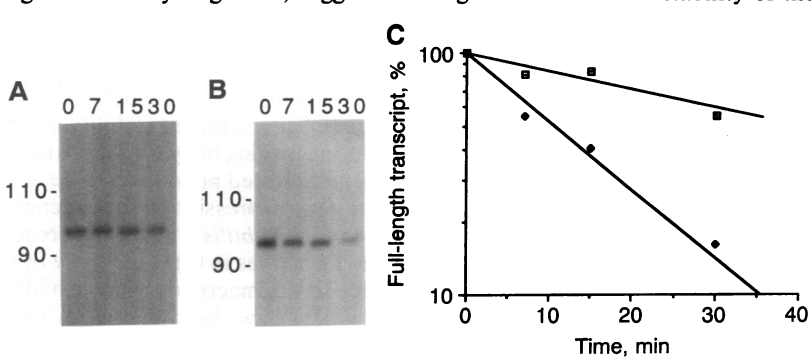

FIG. 4. Half-life of the subtilisin transcript in stationary phase. (*A*) Primer extension analysis of RNA samples taken at T_1 after the addition of 100 μg of rifampicin per ml. (*B*) Primer extension analysis of RNA samples taken at T_3 after the addition of 100 μg of rifampicin per ml. The numbers above the lanes refer to the time in min after rifampicin was added. The primer used for the analysis was that used in Fig. 2*B*. (*C*) Plot of the densitometric analyses of the gels shown in *A* (white squares) and *B* (black squares).

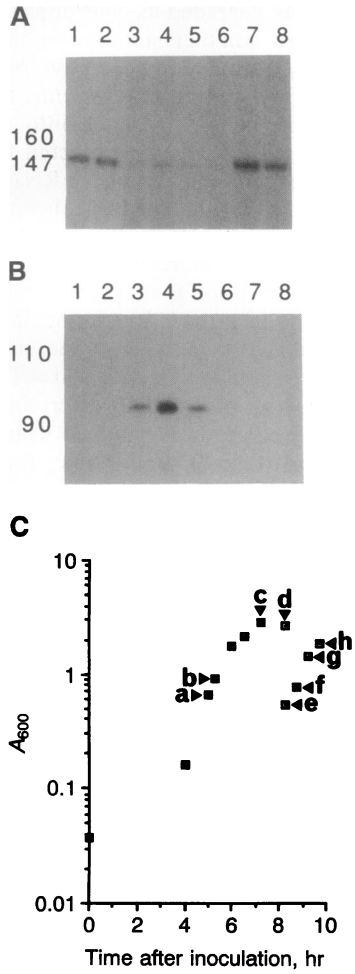

FIG. 5. Cellular concentrations of the SDH and subtilisin transcripts during growth of *B. subtilis* and following dilution of the culture from stationary phase. (*A*) Primer extension analysis of the SDH mRNA. (*B*) Primer extension analysis of the subtilisin mRNA. Lanes 1–8: samples a–h in *C*, which correspond to logarithmic growth, late logarithmic growth, growth at T_1, growth at T_2, growth after the dilution, growth 30 min after dilution, growth 60 min after dilution, and growth 90 min after dilution. The primers used for the analyses were those used in Fig. 2. (*C*) Growth curve and points at which samples were taken for RNA analysis (shown by triangles and letters).

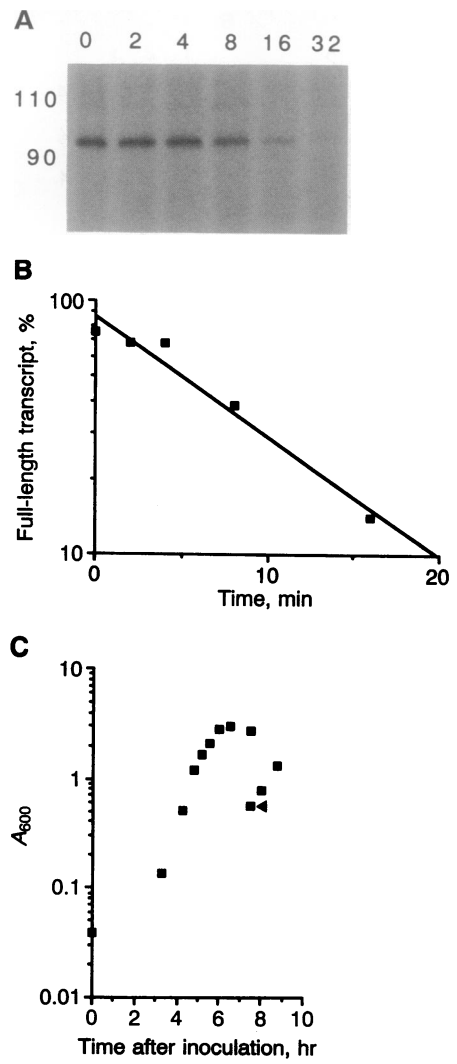

FIG. 6. Disappearance of the subtilisin transcript after dilution of the culture from stationary growth phase. (*A*) Primer extension analysis of RNA samples taken after dilution of the culture. The numbers above the lanes refer to the time in min after the dilution. The primer used for the analysis was that used in Fig. 2*B*. (*B*) Plot of the densitometric analysis of the gel shown in *A*. (*C*) Growth curve and the point at which samples were taken for RNA analysis (shown by a triangle).

When we shifted cells from stationary growth phase back to exponential growth, we saw a rapid disappearance of the subtilisin transcript. From this result we postulate the existence of a mechanism that recognizes the change in growth conditions, and translates this into a change in the cellular concentration of the subtilisin transcript. Further experiments are needed to see whether this mechanism directly or indirectly requires transcription and/or translation. One possible explanation of our results is that there are several mechanisms responsible for regulated mRNA degradation. One type may control the characteristic half-life of a transcript at a given stage in the growth cycle and may be due to stability determinants in the transcript (for a review, see ref. 10). A second type of degradation mechanism may allow organisms to respond rapidly and adjust their gene expression to physiological changes.

In *B. subtilis* there are four examples that we are aware of where the fate of the half-life of specific transcripts was analyzed in response to growth. Two of the transcripts examined are preferentially expressed in vegetative growth (6, L. Melin *et al.*, unpublished data), while the other two are preferentially expressed in postexponential growth (ref. 23 and this work). A comparison of these studies reveals that the transcripts expressed postexponentially can be up to 15 times more stable than those expressed in vegetative growth. In addition the data suggest that, at least in the cases of the transcripts examined, the individual transcripts are more stable when their expression is maximal, and a stability decrease correlates with a decrease in the cellular concentration of the transcripts. Altered rates of mRNA decay are an additional means for cells to control the amount of a specific protein that is made (9). It has been suggested that the general lability of mRNA (3–4 min) in prokaryotes that are growing exponentially allows cells to respond rapidly to physiological changes (24). The increased stability of certain developmental genes during fruiting body formation in *Myxococcus xanthus* has been suggested previously to be a mechanism for increasing the gene expression of those genes (25).

The stability of mRNA in *B. subtilis* is not always regulated in a downward fashion. Two cases in the *erm* family of genes (which confer resistance to the macrolide, lincosamide, and streptogramin B antibiotics) have been shown to increase their mRNA stability in response to erythromycin. The

half-life of the ermA mRNA was shown to increase from 2.5 min to 17.5 min (26), while the ermC mRNA half-life went from 2 min to 40 min (27). The increase in the half-life of both of these transcripts required a 15-min period of induction.

We suggest that the selective stability of transcripts is not limited to prokaryotes. In fact, several observations that substantiate this view have been made in eukaryotes (22, 28, 29). We hope that our findings help to define a model system in which the molecular mechanisms of regulated mRNA stability can be understood.

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