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Inhibition of *Bacillus subtilis aprE* Expression by Lincomycin at the Posttranscriptional Level through Inhibition of ppGpp Synthesis

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Expression of the *Bacillus subtilis* alkaline protease gene *aprE* is controlled by many positive and negative regulators at the transcriptional level. During the course of screening for organic compounds that affect the expression of a translational *aprE*'-'*lacZ* fusion, we found that lincomycin (Lm), erythromycin and chloramphenicol exhibited an inhibitory effect in concentrations that hardly affected cell growth. The antibiotics are known to inhibit protein synthesis by binding to ribosomes. We chose one of them, Lm, for further study. We have previously shown that *aprE* expression requires guanosine 3′,5′-bisdiphosphate (ppGpp) synthesized on the ribosome by the stringent factor RelA. An examination of Lm-treated cells showed that the levels of ppGpp were greatly reduced in these cells, and the inhibitory effect of the antibiotic was not seen in *relA*-disruption mutants. Transcriptional levels of *aprE*, however, were not influenced by Lm treatment as shown by using a transcriptional *aprE-lacZ* fusion as well as quantitative RT-PCR. Furthermore, disruption of *relA* did not affect the expression of transcriptional *aprE-lacZ*. From these results, we conclude that *aprE* expression is controlled by the stringent control at the posttranscriptional level, and that Lm inhibits this process by inhibiting ppGpp synthesis on the ribosome.

Key words: *Bacillus subtilis aprE*, lincomycin, ppGpp, posttranscriptional regulation, RelA, stringent control.

Abbreviations: Cm, chloramphenicol; Cm^r, chloramphenicol resistance; Em, erythromycin; Em^r, erythromycin resistance; Km, kanamycin; Km^r, kanamycin resistance; Lm, lincomycin; Rf, rifampicin; BS, blasticidin S; Tc, tetracycline; Sm, streptomycin; MC, mitomycin C; ppGpp, guanosine 3′,5′-bisdiphosphate.

Bacillus subtilis produces two major extracellular proteases, the alkaline and neutral proteases, whose production begins after the cessation of exponential growth. The expression of *aprE* encoding the extracellular alkaline protease is regulated at the transcriptional level by both positive and negative regulators, and among these regulators the DegS-DegU two-component system and Spo0A play major roles (*1–3*). It is thought that upon signal input to induce the expression of *aprE*, the DegS kinase undergoes ATP-dependent autophosphorylation and transfers the phosphate to DegU, which results in the transcriptional activation of the *aprE* gene. Although in vitro phosphorylation and dephosphorylation of the DegS and DegU proteins have been well documented (*4–6*), what actually triggers the phosphorylation reaction and how phosphorylated DegU stimulates the transcription of *aprE* remain largely unknown. It is likely that, in its natural habitat, a *Bacillus subtilis* cell secretes exoproteases upon depletion of intracellular amino acids or nitrogen sources, as they are synthesized after the cessation of exponential growth phase. It is possible that the intracellular state of nitrogen sources in the cell is caught by DegS and transmitted to DegU, since DegS lacks a predicted hydrophobic, membrane-spanning domain, and, therefore, is presumed to be a cytosolic protein. In addition to the two major factors, it has been demonstrated that the stringent factor RelA is an important positive factor for the expression of *aprE*, indicating that guanosine 3′,5′-bisdiphosphate (ppGpp) is involved in *aprE* expression (*7*). That ppGpp is an intracellular substance that directs the transcription of genes involved in a large number of cellular activities (*8*) suggests again that the intracellular state of the cell submits a signal that induces the expression of *aprE*.

Antibiotics with known modes of action have often been shown to be useful as molecular probes for mechanistic studies on molecular events in the cell. Thus, the use of antibiotics would also contribute to the understanding of *aprE* expression. In this study we screened for inhibitors that affect *aprE* expression and found that protein synthesis inhibitors such as lincomycin (Lm), chloramphenicol (Cm) and erythromycin (Em) inhibit the expression of a translational *aprE'-'lacZ* fusion. We demonstrate that the inhibitory effect of Lm on *aprE* expression is exerted at the posttranscriptional level through the inhibition of ppGpp synthesis by RelA.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Media—The following *B. subtilis* strains were use: CU741 [*trpC2 leuC7*], YY102 [*trpC2 leuC7 aprE'-'lacZ* (translational fusion; kanamycin resistance, Km^r)], HT1013 [*trpC2 leuC7 relA333 aprE'-'lacZ* (translational fusion; Km^r)], HT1020 [*trpC2 degU32*(Hy)

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aprE'-'lacZ (translational fusion; Km^r)] (*4*, *7*). *B. subtilis* MAPCM1131 [*trpC2 leuC7 aprE-lacZ* (transcriptional fusion; chloramphenicol resistance, Cm^r)] was constructed by the insertion of plasmid pMUAPR11 (see below) into strain CU741. The expression of *aprE-lacZ* thus constructed was stimulated by multicopy *degQ* (*9*), indicating that the fusion is under the control of the *aprE* promoter. *B. subtilis* MAPREL41 [*trpC2 leuC7 aprE-lacZ* (transcriptional fusion; Cm^r) *relA333*] was constructed by transformation of strain MAPCM1131 with DNA prepared from strain HT1013. Luria-Bertani (LB) medium was from Difco Laboratories. Schaeffer's and Spizizen's minimal media were prepared as described previously (*11*, *12*). X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside) was added where appropriate at a concentration of 100 μg/ml.

Plasmids—A pHP13 derivative, pHB201, specifying erythromycin resistance (Em^r) and Cm^r was obtained from the Bacillus Genetic Stock Center (Cleveland, Ohio) (*10*). Plasmid pMUCM2 was constructed by the insertion of a *Sma*I fragment carrying Cm^r from pBEST4C (*13*) between the two *Sca*I sites in pMutinII (*14*). To create pMUAPR11, the PCR fragment obtained by using primers APRE20F: 5′-AGTTGAATTCGATCAGCTTGTTGTTTGCGTTA-3′ and APRE310R: 5′-AGTTAGATCTGTGCAATATGATCTTCTTCCACAT-3′ was cleaved with *Eco*RI and *Bgl*II, and inserted between the *Eco*RI and *Bam*HI sites of pMUCM2.

Antibiotics—The antibiotics lincomycin (Lm), streptomycin (Sm), rifampicin (Rf), tetracycline (Tc), and mitomycin C (MC) were obtained from Sigma Chemical (St. Louis, MO). Blasticidin S (BS) was a gift from S. Aizawa. The other antibiotics used for screening were either the gifts from T. Sato or obtained from Sigma Chemical.

Measurement of ppGpp Concentration in B. Subtilis Cells—Two milliliters of an overnight culture of strain YY102 in LB medium was inoculated into 50 ml of Spizizen's minimal salt medium supplemented with glucose (1%), casamino acids (1%) and tryptophan (50 μg ml^{-1}). Cells were grown to midlog phase and collected on a membrane filter (0.45 μm, 90 mm; Toyo Roshi, Tokyo). The filter was cut into 6 pieces, which were then separately suspended in 20 ml of prewarmed Spizizen's minimal salt containing varying concentrations of Lm. After incubation for 10 min at 37°C, the cells were collected on filters (0.45 μm, 47 mm; Millipore), immediately suspended in 1 N formic acid at 0°C and gently shaken for 60 min. After centrifugation, the supernatants were filtered (0.2 μm, 25 mm; Millipore) and lyophilized. The lyophilized materials were dissolved in distilled water and subjected to high-performance liquid chromatography (HPLC) on a system equipped with a Partisil-10 SAX column (GL Science) as described previously (*15*). Concentrations of ppGpp in the cell are expressed as picomoles per OD600 nm per milliliter.

β-Galactosidase Assay—*B. subtilis* cells were grown overnight in LB medium, and inoculated into Schaeffer's sporulation medium containing the antibiotics tested. Determination of β-galactosidase activities was carried out twice or more as described previously (*16*). Values thus obtained did not vary by more than 15%, and representative results are shown in this paper.

RNA Isolation and RT-PCR—Cells of strain CU741 were grown in Schaeffer's medium (50 ml) with and without Lm, and collected by centrifugation. Total RNA was isolated from the cells as described previously (*17*). cDNA was synthesized and amplified by using PCR primers APRF64: 5′-TTCAGCAACATGTCTGTGCAG-3′ and APRR383: 5′-GTGTAGCCTTGAGAGTGAAG-3′, and a Real Time One Step RNA PCR KIT (Takara Biomedicals, Shiga) by the procedure recommended by the supplier. The DNA amplification step was monitored by a Smart Cycler System (Cepheid, USA) with SYBR Green I, and the relative concentrations of mRNAs were estimated from the cycle threshold (C_t), which was defined as the first cycle in which there is a significant increase in fluorescence above the background level. RT-PCR was performed for two concentrations of each RNA sample (1 μg and 100 ng), and similar results were obtained in both cases. Under the conditions used, the C_t values determined for rRNAs used as controls in various RNA samples were indistinguishable. The PCR products synthesized by this procedure were verified by melting curve analysis in the Smart Cycler System apparatus and by agarose gel electrophoresis for size determination.

RESULTS

Effects of Antibiotics on Translational aprE'-'lacZ Expression—To screen for inhibitors that might affect the expression of *aprE*, we devised a simple method by which to identify such a phenotype. *Bacillus subtilis* YY102 carrying a translational *aprE'-'lacZ* fusion was grown overnight in LB medium, and spread on Schaeffer's sporulation medium plates containing X-gal, and paper discs impregnated with various compounds were placed on the plates. After overnight incubation at 37°C, the plates were examined for cell growth and color development. We tested more than 80 compounds including amino acids, nucleosides, surface active agents, and antibiotics inhibiting DNA, RNA, protein and cell wall syntheses, those

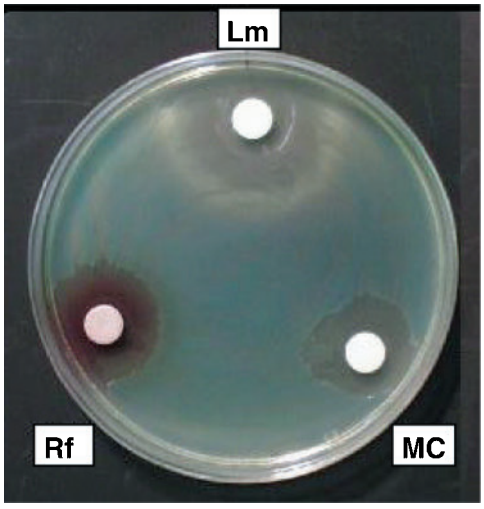

Fig. 1. **Effect of Lm, Rf and Mc on the expression of a translational *aprE'-'lacZ* fusion and cell growth of *B. subtilis* YY102.** Cells grown overnight in LB medium were diluted 100 times, and 0.1 ml was spread on an LB agar plate containing 100 μg/ml of X-gal. Paper discs containing antibiotics were placed on the agar plate, which was then incubated over night at 37°C.

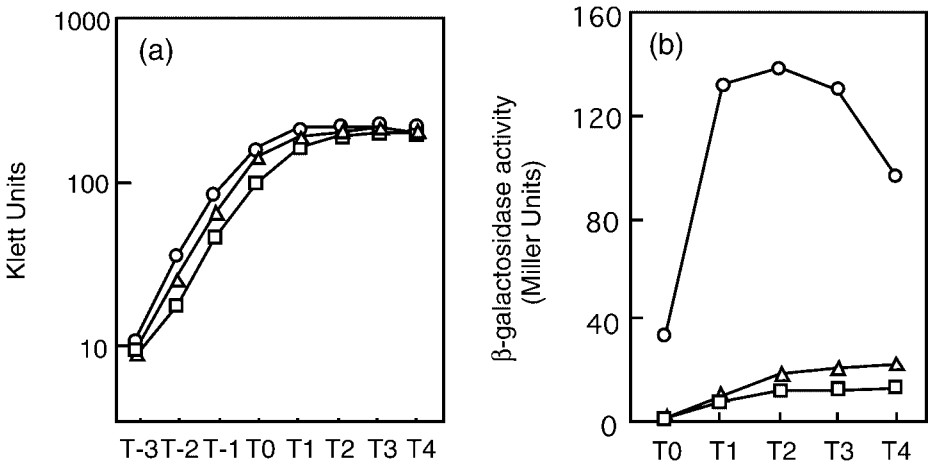

Fig. 2. **Effect of Lm on the expression of a translational *aprE'-'lacZ* fusion.** (a) growth. (b) β-galactosidase activity. Strain *B. subtilis* YY102 (*aprE'-'lacZ*) was grown in Schaeffer's sporulation medium with or without Lm, and the cells were harvested at hourly intervals after the cessation of logarithmic growth for the measurement of β-galactosidase activity. Growth was monitored by a Klett-Summerson spectrophotometer with the red filter. Numbers on the x-axis represent the growth time in hours relative to the end of vegetative growth (T0). Symbols: circles, 0 μg/ml; triangles, 1.25 μg/ml; squares, 5.0 μg/ml.

which interfere with membrane integrity, and those whose mode of action have not been determined. Two different growth patterns were observed depending on the compounds used: one showing a circular inhibition zone surrounded by a blue lawn of cells, and the other exhibiting a white zone of cell growth between the inhibition zone and the blue lawn of cells. A typical experiment is shown in Fig. 1, in which Lm, Rf and MC were used. The blue lawn and the white zone are interpreted to contain cells in which *aprE'-'lacZ* expression was fully induced and prevented, respectively. By this screening program, we found that only the protein synthesis inhibitors, including Lm, Em, Cm, BS, Tc, but not Sm, showed the latter growth pattern.

We performed quantitative analyses on the effects of Lm, Em, Cm and Rf on *aprE'-'lacZ* expression using YY102 cells grown in liquid Schaeffer's medium. The expression profile of *aprE'-'lacZ* in the presence of Lm is shown in Fig. 2. Within the concentrations that showed little or no growth inhibition (Fig. 2a), the expression of *aprE'-'lacZ* was greatly reduced (Fig. 2b). The other two antibiotics, Em and Cm, also prevented *aprE'-'lacZ* expression at concentrations that had no or only a marginal effect on cell growth, whereas Sm and Rf did not show an inhibitory effect even at concentrations that retarded cell growth (Table 1). These results are consistent with the growth patterns seen on the plate assay (data not shown). The antibiotics Lm, Em and Cm inhibit procaryotic protein synthesis by binding to ribosomes (*18–20*). Rf is a specific inhibitor of bacterial RNA polymerase (*21*). Tc and BS are inhibitors of protein synthesis (*22*, *23*), and from the growth pattern of YY102 on X-gal-containing plates, these antibiotics are also likely to inhibit *aprE'-'lacZ* expression at sub-inhibitory concentrations. These results led us to conclude that ribosomes are involved in *aprE* expression. It should be noted that Sm binds to ribosomes and inhibits protein synthesis (*24*), but does not show an inhibitory effect on *aprE'-'lacZ* expression (Table 1). This must be due to a difference in the mode of action of Sm from those of the protein synthesis inhibitors described above (see also Discussion): Sm does not affect the growing peptide bond synthesis (*25*, *26*, and original references therein), and the polysome level decreases in cells treated with Sm, which leads to an accumulation of monosomes that are incapable of protein synthesis.

Table 1. **Effect of antibiotics on aprE'-'lacZ expression in wild-type and lincomycinresistant strains.**

Strain	Antibiotics (μg/ml)	Concentration	Expression level* (% of control)
YY102	No antibiotic	0	100
	Lincomycin	1.25	16
		5.0	10
	Erythromycin	0.05	20
		0.1	10
	Chloramphenicol	0.25	48
		0.5	30
	Streptomycin	2.5	129
		5.0	135
	Rifampicin	0.005	123
YY102 (pHB201)	Lincomycin	5.0	115

Strains *B. subtilis* YY102 (*aprE'-'lacZ*) and its pHB201-carrying derivative were grown in Schaeffer's sporulation medium, and β-galactosidase activities were determined at hourly intervals as described in "EXPERIMENTAL PROCEDURES." *The numbers indicate the values expressed as percent of the β-galactosidase activity obtained without the addition of the antibiotics. Mean values of the highest β-galactosidase activities observed between T2 and T4 from two experiments were taken for calculation. The variations of the peak values were within 15%. The control value without the addition of the antibiotics was 150 Miller units.

We selected Lm for further study, since it exhibited a large concentration difference in the inhibition of *aprE'-'lacZ* expression and cell growth (Fig. 1 and Table 1). If the Lm effect on *aprE* expression is through the inhibition of ribosomal function, the inhibitory effect should not be observed when a strain carrying a ribosomal mutation to Lm resistance is used. Plasmid pHB201 carries the *erm* gene that specifies 23S rRNA methylase causing resistance to Em, Lm and streptogramin B (MLS) antibiotics (*27*). It was found that in pHB201-carrying cells, the expression of *aprE'-'lacZ* was not affected by Lm (Table 1), suggesting that the antibiotic inhibits *aprE'-'lacZ* expression through its effect on ribosomal function.

Inhibition of ppGpp Synthesis by Lincomycin—Since Lm inhibits ribosomal function by binding to ribosomes, the results described so far may indicate that *aprE* expression is influenced by ribosomes or some factor

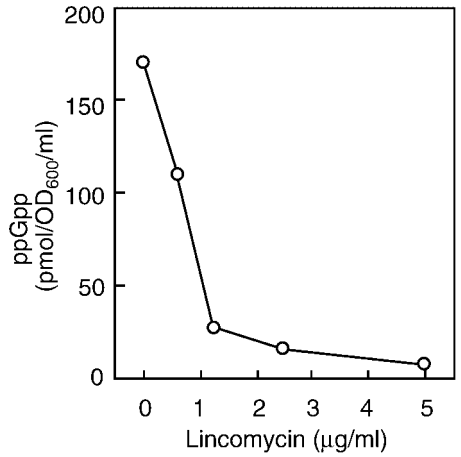

Fig. 3. **Inhibition of ppGpp synthesis by Lm in strain YY102.** Experimental conditions are described in "EXPERIMENTAL PROCEDURES."

associated with them. It is known that in addition to their major role in protein synthesis, ribosomes together with the stringent factor RelA, respond to the depletion of amino acids, nitrogen and carbon sources, and energy, by producing ppGpp (*8*). This results in a change in the gross transcriptional state in an emergency. We have shown that the efficient expression of *aprE* requires the stringent factor RelA: *aprE* expression is greatly reduced by *relA* disruption (*7*). These results suggest that Lm might inhibit *aprE* expression through the inhibition of ppGpp synthesis. To test this possibility, we examined the effect of Lm on ppGpp synthesis in *B. subtilis* cells. We used nutritional shift-down culture to determine the ppGpp level (see "EXPERIMENTAL PROCEDURES"), because the nucleotide content was found to be much higher in cells from nutritional shift-down culture than in those from culture in Schaeffer's sporulation medium, and thus any difference in the ppGpp level would be more accurately determined by this procedure. As shown in Fig. 3, Lm inhibited ppGpp synthesis in a concentration-dependent manner, and the concentrations at which Lm exerted its inhibitory effect corresponded well to those that prevented *aprE'-'lacZ* expression (Table 1).

Effect of Lincomycin on aprE'-'lacZ Expression in relA Mutant—We have previously shown that the expression level of *aprE'-'lacZ* in a *relA* disruption mutant was reduced to 11–15% of the wild type level in both a *degU*$^+$ strain and a *degU32*(Hy) mutant, an overproducer of the exoproteases (*7*). If the effect of Lm on *aprE'-'lacZ* expression is correlated with the inhibition of ppGpp synthesis, it is expected that the residual *aprE'-'lacZ* activity seen in the *relA* mutant would not be affected by Lm. As shown in Fig. 4, the addition of Lm did not appreciably affect the low-level expression of *aprE'-'lacZ* in *relA333 degU*$^+$ cells (Fig. 4a) as well as the high-level expression of *aprE'-'lacZ* in the *relA333 degU32*(Hy) cells (Fig. 4b). It should be noted that there was no difference in the minimal inhibitory concentrations of Lm toward the two mutant strains and their wild-type strains (data not shown). Also the results shown in Fig. 4 deny the possibility that the translation apparatus for *aprE* mRNA is particularly sensitive to Lm and that the reduction in *aprE'-'lacZ* expression by the antibiotic (Table 1 and Fig. 2) is due to the inhibition of translation of the *aprE* mRNA. These results indicate a strong correlation between the inhibition of ppGpp synthesis by Lm and the decreased expression of *aprE*.

Involvement of Stringent Control in the Posttranscriptional Expression of aprE—In the experiments so far described, we used a translational *aprE'-'lacZ* fusion. To examine the effect of Lm on the transcription of *aprE*, we measured β-galactosidase activities derived from a transcriptional *aprE-lacZ* fusion that we constructed (see "EXPERIMENTAL PROCEDURES"). The *lacZ* gene in the translational *aprE'-'lacZ* fusion is flanked by the ribosome-binding site (RBS) site and the start codon of *aprE*, whereas that in the transcriptional *aprE-lacZ* fusion is flanked by the RBS and the start codon of *B. subtilis spoVG* (*7*, *14*). As shown in Fig. 5a, no inhibition of *aprE-lacZ* expression was seen at Lm concentrations that severely inhibit the expression of translational *aprE'-'lacZ* fusion (see Fig. 2). Furthermore, quantification of mRNA levels of *aprE* by RT-PCR did not show a significant difference between the Lm-treated and control cells (Fig. 5b). It is known that in *E. coli* the synthesis of abundant RNA such as rRNA or tRNA in *relA* mutants does not stop even after the mutant cells enter stationary

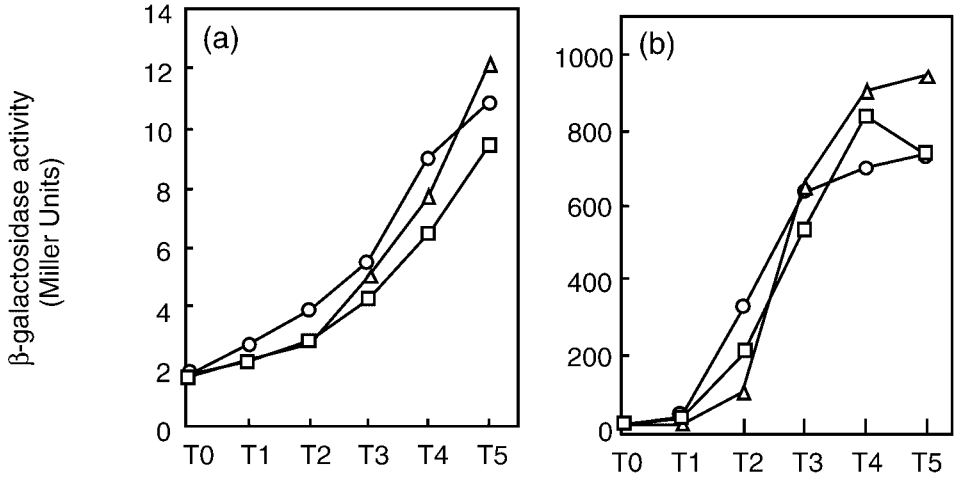

Fig. 4. **Effect of Lm on the expression of a translational *aprE'-'lacZ* fusion in strains carrying a *relA* mutation.** (A) HT1013 *aprE'-'lacZ relA333*. (B) HT1020 *aprE'-'lacZ relA333 degU32*(Hy). Symbols: circles, 0 μg/ml; triangles, 1.25 μg/ml; squares, 5.0 μg/ml.

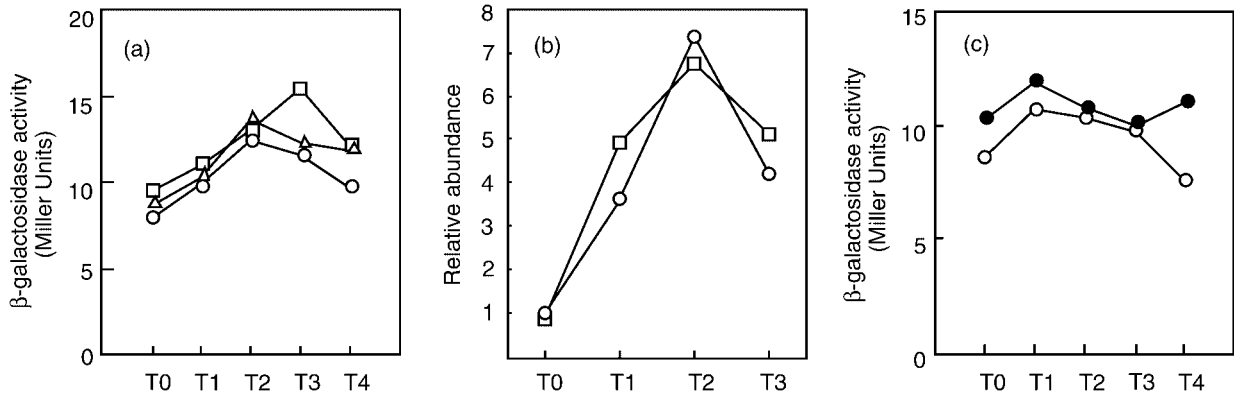

Fig. 5. **Effect of Lm and the *relA333* mutation on the transcription of *aprE* as determined by an *aprE-lacZ* transcriptional fusion (a, c) and RT-PCR (b).** (a) Strain MAPCM1131 was grown in Schaeffer's medium, and β-galactosidase activities were determined for the cells harvested at the indicated times. Lm concentrations, circles, 0/ μg ml; triangles, 1.25 μg/ml; squares, 5.0 μg/ml. (b) RT-PCR. Preparation of RNA from strain CU741 grown in the presence or absence of Lm, and the procedure for RT-PCR are described in "EXPERIMENTAL PROCEDURES." The data shown here are those from experiments in which 1 μg of the RNA samples were used. Lm concentrations, circles, 0 μg/ml; squares, 5.0 μg/ml. (c) Growth conditions and the determination of β-galactosidase activity were the same as those described in (a) except for the strains used. solid circles, MAPCM1131 *relA*$^+$; open circles, MAPREL41 *relA333*.

phase (*8*). This would result in a relative decrease in the levels of mRNAs whose synthesis is not under stringent control as compared with those of the abundant RNAs. The observation that the *aprE* mRNA levels were similar in both *relA* and wild-type cells (Fig. 5b) may be due to the increased expression of *aprE*, which is under positive stringent control (*7*).

The results with Lm described above strongly suggested that a defect in ppGpp synthesis results in the posttranscriptional, but not transcriptional, expression of *aprE*. To test this possibility, we examine the expression of the transcriptional *aprE-lacZ* fusion in both *relA* and wild-type strains. The results depicted in Fig. 5c show that, in contrast to the observation on translational *aprE'-'lacZ* fusion (*7*), the expression of transcriptional *aprE-lacZ* fusion is not affected by *relA* disruption. These results show that the regulation of *aprE* expression by ppGpp is exerted at the posttranscriptional level.

DICUSSION

Low molecular-weight compounds with known modes of action are often useful for the elucidation of various cellular processes. In this study we looked for compounds that affect the expression of *aprE* using a translational *aprE'-'lacZ* fusion in *B. subtilis* cells. It was shown that among the compounds tested only a group of protein synthesis inhibitors that include Lm, Em and Cm (Table 1), and probably BS and Tc as well, has such inhibitory activities. Further studies with Lm showed that it inhibits ppGpp synthesis (Fig. 3), and that no inhibition of *aprE'-'lacZ* expression by Lm was observed in a *relA* (*relA333*) mutant in which ppGpp synthesis is negligible (Fig. 4). We have previously shown that the inhibition of ppGpp synthesis by the *relA333* mutation results in a reduction in *aprE'-'lacZ* expression (*7*). From these results we conclude that Lm prevents *aprE* expression by inhibiting ppGpp synthesis on the ribosome. Lund and Kjeldgaad (*28*) have shown that Em and Cm exert an inhibitory effect on ppGpp synthesis in *E. coli*. Thus, it is likely that the two antibiotics also inhibit *aprE'-'lacZ* expression through the inhibition of ppGpp synthesis.

It was shown with transcriptional and translational fusions and RT-PCR that the inhibition of *aprE* expression by Lm is at the posttranscriptional level (Fig. 2 and Fig. 5, a and b). A further study revealed that the upregulation of *apr*E by ppGpp is also exerted at the posttranscriptional level (Fig. 5c). In most cases so far reported, ppGpp regulates transcription during stationary phase by binding to RNA polymerase (*8*). The regulation of gene expression at the posttranscriptional level, however, is not unprecedented. In an earlier work by Williams and Rogers it was shown that a large amount of the *argH* gene product accumulated in *E. coli relA*$^+$ but not in *relA* cells, whereas the synthesis and stability of *argCBH* mRNA was largely unchanged (*29*). Another example of posttranscriptional regulation by ppGpp is that reported for the *E. coli rpoS* gene (*30–33*). The gene specifies the σ^s subunit of RNA polymerase, which controls the expression of a large number of genes that are induced during entry into stationary phase. The finding that *aprE* is under the posttranscriptional control of ppGpp suggests an interesting question of what the target of ppGpp is in the process after the transcription of *aprE*. Stability of the mRNA may be excluded, since the levels of mRNA determined by RT-PCR were similar in both the wild-type and *relA* cells (Fig. 5b). The known regulators of *aprE* expression so far reported are in every case involved in transcription (*1–3*). Therefore, this is the first example of *aprE* expression that is regulated at the posttranscriprional level.

Studies on the synthesis of heat shock proteins by subjecting the host cells to translational blocks led Van-Bogelen and Neidhardt to propose that the ribosome serves as a sensor of heat and cold shock (*34*). They classified protein synthesis inhibitors into two groups based on the proteins induced after treatment with antibiotics. One group (H-group), including Sm and Km, render the cell competent to synthesize cellular proteins similar to those seen in the heat shock response, while the other (C-

group), including Cm and Em, exhibit protein patterns similar to those observed in cold shock response. C-group antibiotics inhibit the peptidyl tranferase reaction on the ribosome, which would result in the occupation of a charged tRNA at the A-site, a situation similar to that seen when the ribosome senses cold shock and slows down the peptidyl transferase reaction (*34*, *35*). It has been suggested that the resultant high concentrations of charged tRNA at the A-site may block the synthesis of ppGpp by RelA. Since Lm inhibits protein synthesis by blocking the peptidyl transferase reaction (*18*), it is possible that a reduction in the ppGpp level by the antibiotic (Fig. 4) causes the inhibition of *aprE* expression. BS and Tc show growth patterns on the assay plates similar to those shown by Lm, Cm and Em (results not shown). BS inhibits peptidyl transferase (*23*), possibly leaving charged tRNA at the A site, suggesting that the antibiotic belongs to the C group and affects ppGpp synthesis. Tc, on the other hand, was shown to inhibit ppGpp synthesis in the absence of ribosomes (*36*). It is possible that the inhibition of RelA by these antibiotics causes the inhibition of *aprE* expression.

The previous report (*7*), together with the current results, shows that *aprE* expression is under stringent control, indicating that *aprE* expression is regulated by at least three major regulatory pathways, *i.e.*, stringent control, and the Spo0A-AbrB and DegS-DegU systems (*1*). Presumably the repression of *aprE* by AbrB is released by Spo0A at the onset of stationary phase, and *aprE* expression may be fully induced if ppGpp and phosphorylated DegU are available in the cell. A deficiency of amino acids in the cell would serve as a signal that leads to *aprE* expression, since the lack of amino acids results in ppGpp synthesis (*8*), and this nucleotide stimulates *aprE* expression.

It has been demonstrated in *Salmonella typhimurium* that subinhibitory concentrations of antibiotics, including Em, activate or repress the transcription of many genes, which is distinct from their growth-inhibitory effects (*37*). They suggest that the transcription machinery is coupled to macromolecular processes, and, thus, transcription is affected by the binding of non-growth-retarding concentrations of antibiotics to their macromolecular targets. With the results described here, it seems likely that their observation with Em is due at least in part to the inhibition of ppGpp synthesis by the antibiotic. In this respect, we note that a deletion of the *relA* gene in *B. subtilis* leads to profound effects on gene expression (*38*).

Antibiotics showing inhibitory effects on ppGpp synthesis might confer an advantage on antibiotic-producing host cells. Since ppGpp-dependent stringent control may be a means of fighting adverse environments, a reduction in the ppGpp level will be disadvantageous in terms of competition with other microorganisms. Upon contact of a cell with subinhibitory concentrations of an inhibitor of ppGpp synthesis produced by another microorganism, the level of ppGpp in the former cell would be reduced. This would result in a failure of the stringent response and cause a superfluous synthesis of macromolecules or energy production, which is thought to be disadvantageous for survival in nature.

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