JOURNAL OF BACTERIOLOGY, Dec. 1988, p. 5642–5646
0021-9193/88/125642-05$02.00/0

Vol. 170, No. 12

Induction of *cat-86* by Chloramphenicol and Amino Acid Starvation in Relaxed Mutants of *Bacillus subtilis*

NICHOLAS P. AMBULOS, JR., ELIZABETH J. ROGERS, ZLATKA ALEXIEVA, AND PAUL S. LOVETT*

Department of Biological Sciences, University of Maryland Baltimore County, Catonsville, Maryland 21228

Received 19 July 1988/Accepted 12 September 1988

The chloramphenicol acetyltransferase gene *cat-86* is induced through a mechanism that is a variation of classical attenuation. Induction results from the destabilization of an RNA stem-loop that normally sequesters the *cat-86* ribosome-binding site. Destabilization of the stem-loop is due to the stalling of a ribosome in the leader region of *cat-86* mRNA at a position that places the A site of the stalled ribosome at leader codon 6. Two events can stall ribosomes at the correct location to induce *cat-86* translation: addition of chloramphenicol to cells and starvation of cells for the amino acid specified by leader codon 6. Induction by amino acid starvation is an anomaly because translation of the *cat-86* coding sequence requires all 20 amino acids. To explain this apparent contradiction we postulated that amino acid starvation triggers intracellular proteolysis, thereby providing levels of the deprived amino acid sufficient for *cat-86* translation. Here we show that a mutation in *relA*, the structural gene for stringent factor, blocks intracellular proteolysis that is normally triggered by amino acid starvation. The *relA* mutation also blocks induction of *cat-86* by amino acid starvation, but the mutation does not interfere with chloramphenicol induction. Induction by amino acid starvation can be demonstrated in *relA* mutant cells if the depleted amino acid is restored at very low levels (e.g., 2 μg/ml). A mutation in *relC*, which may be the gene for ribosomal protein L11, blocks induction of *cat-86* by either chloramphenicol or amino acid starvation. We believe this effect is due to a structural alteration of the ribosome resulting from the *relC* mutation and not to the relaxed phenotype of the cells.

The stringent response in bacteria involves a reduction in net RNA synthesis due to the depletion of a required amino acid (4). The mechanism responsible for this general phenomenon has been partially determined, largely through in vivo and in vitro studies with *Escherichia coli*. Deprivation of a single amino acid results in the accumulation of a class of uncharged tRNA molecules within the cell. When such an uncharged tRNA species enters the A site on a translating ribosome the *relA* gene product, stringent factor, interacts with a protein(s) in the ribosome (which includes the *relC* product), causing ribosomes to synthesize primarily ppGpp (magic spot I) and, to a lesser extent, pppGpp (magic spot II). These nucleotides block transcription of several operons, including those for rRNAs and tRNAs. Although this description of the stringent response is certainly an oversimplification of a complex mechanism (4), it serves to demonstrate the central role of the bacterial ribosome in the process.

Studies of the induction of the chloramphenicol acetyltransferase (CAT) gene *cat-86* in *Bacillus subtilis* have also demonstrated that the bacterial ribosome is central to the induction process. Induction is due to changes in the secondary structure of *cat-86* mRNA due to ribosome stalling (10). A ribosome stalled at a precise site in the leader region of *cat-86* mRNA destabilizes a stem-loop structure that normally sequesters the *cat-86* ribosome-binding site (1). Thus, ribosome stalling activates translation of *cat-86* mRNA (6). *cat-86* mRNA translation is typically activated by stalling ribosomes in the leader through the action of chloramphenicol, the classic inducer of *cat* genes of gram-positive origin (10). However, ribosomes that are stalled at the correct location in the *cat-86* leader by starving host cells for a leader-encoded amino acid also induce translation of *cat-86* mRNA (5). It is evident, therefore, that the focus of the *cat-86* induction mechanism is the ribosome itself.

To further assess the role of the ribosome in *cat-86* induction, we examined the effects of two well-characterized mutations that alter the stringent response in *B. subtilis*. Our results are consistent with the central role proposed for the bacterial ribosome in *cat-86* induction. The data also support a previously suggested explanation to account for the successful translation of *cat-86* mRNA during induction by amino acid starvation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used are listed in Table 1. The plasmids used were pPL703 and pPL703-Thr. pPL703 is a promoter-cloning plasmid in which the promoter-deficient reporter gene is *cat-86*, which specifies CAT (12). In all experiments described *cat-86* was activated by the Spac promoter-containing fragment (21). The replicon of pPL703 is derived from pUB110, and the pUB110 sequences in pPL703 also provide a neomycin resistance gene that serves as a neutral selection marker for pPL703. pPL703-Thr is identical to pPL703, except that the first lysine codon (AAA) in the *cat-86* leader was changed, by site-directed mutagenesis, to a threonine codon (ACA) (Fig. 1) (5). Throughout this report we do not refer to the plasmids, but rather we refer to the version of *cat-86* under study. pPL703 contains *cat-86*, whereas pPL703-Thr contains *cat-86* Thr.

Induction of *cat-86* or *cat-86* Thr expression by chloramphenicol (2 μg/ml) or amicetin (0.5 μg/ml) was performed for 2 h with log-phase cells grown in Penassay broth. Induction by amino acid starvation was accomplished by growing *cat-86* (or *cat-86* Thr)-containing cells to the mid-log phase in a minimal medium supplemented with all amino acids (each at 50 μg/ml) (5). Cells were washed with cold, unsupplemented minimal medium and suspended in the same medium

* Corresponding author.

TABLE 1. Strains of *B. subtilis*

Strain	Genotype	Reference or source
BR151	*lys-3 metB10 trpC2*	5
BD54	*leu met lys*	D. Dubnau
ZA101	*leu lys-3 metB10*	This paper[a]
IS56	*lys-3 trpC2 relA*	17–19
IS58	*lys-3 trpC2*	17
IS169	*trpC2 phe relC*	18

[a] Constructed by congression: DNA from BD54 was transformed into BR151 at 5 μg/ml. Trp^+ transformants were selected, and a leucine-requiring transformant was identified.

containing all amino acids except lysine. The cells were starved for lysine during a 2-h incubation at 37°C, and CAT specific activity was measured.

CAT specific activity. CAT was measured by the colorimetric procedure of Shaw (15), and protein was determined by the Bradford method (3). CAT specific activity is reported as the number of micromoles of chloramphenicol acetylated per minute per milligram of protein at 25°C.

Genetic procedures. Plasmids were transformed into cells made competent by the Bott and Wilson method (2). PBS1 transduction was as previously described (13). Methods for plasmid isolation and purification have been described in detail (11).

RESULTS

***cat-86* induction in a *relA* mutant of *B. subtilis*.** Bacterial mutants that do not exhibit the stringent response during amino acid starvation are said to be relaxed (4). In *B. subtilis* mutations at any of three loci confer the relaxed, or Rel, phenotype (9). The *relA* gene is thought to be the structural gene for stringent factor, and the *relC* gene is believed to encode a ribosomal protein whose function is essential to the synthesis of ppGpp (17, 18). The *relG* locus is less well defined, but this gene appears to be essential for the stringent response induced by glucose starvation of *B. subtilis* (14). The *relG* mutation was not examined in the present study.

cat-86 and the mutant derivative *cat-86* Thr were introduced by transformation into strains IS56 (*relA*), IS58, and BR151. The latter two strains are Rel^+, and IS58 is largely isogenic with IS56. *cat-86* Thr was typically used for experiments in which *cat-86* expression was induced by lysine starvation. *cat-86* Thr gives an enhanced level of induction during lysine deprivation, because in this mutant gene the leader contains only one lysine codon and this is present at codon 6 (5). In the wild-type *cat-86* gene a lysine codon is represented twice in the leader, at codons 3 and 6 (Fig. 1).

The *relA* mutation in IS56 did not alter induction of *cat-86* by chloramphenicol (Fig. 2) or by the alternative inducer amicetin (data not shown) (7). By contrast, induction of *cat-86* Thr or the wild-type *cat-86* gene by lysine deprivation could not be demonstrated in the *relA* mutant host (Fig. 2). To test whether the host defect that blocked starvation-mediated induction was the *relA* mutation, we took advantage of the observation that *relA* is about 60% linked by PBS1 transduction to the leucine operon (17). *cat-86* Thr was transformed into the leucine-requiring *B. subtilis* mutant ZA101. PBS1 grown on strain IS56 (*relA*) was used to transduce ZA101 (*cat-86* Thr) to Leu^+. Thirty-eight transductants were purified by single-colony isolation, and each was tested for induction of the resident *cat-86* Thr gene by lysine deprivation. Each transductant was also tested for RNA synthesis ($[^3H]$uracil incorporation) 5 min after lysine deprivation. Nineteen of the 38 transductants (i.e., 50% linkage with *leu*) failed to support induction of *cat-86* by lysine deprivation, and these same transductants were judged to be *relA* mutants since they were highly active in RNA synthesis during starvation for lysine (Table 2). The remaining transductants supported *cat-86* Thr induction by lysine deprivation, and these were judged to be $relA^+$ because they failed to synthesize significant levels of RNA during lysine starvation. All of the transductants permitted induction of *cat-86* Thr by chloramphenicol, although the induction was consistently low. This is in agreement with our previous report, in which it was shown that *cat-86* Thr did not induce as well as *cat-86* with either chloramphenicol or amicetin as the inducer (5).

To confirm that the apparent correlation between *relA* transduction and the loss of *cat-86* induction by lysine deprivation was not an artifact, we repeated the above transduction experiment but selected for Met^+ transductants of ZA101 (*cat-86* Thr). *metB10* and *relA* should not link by PBS1 transduction (9); indeed, each of six transductants tested failed to significantly incorporate $[^3H]$uracil after lysine deprivation, and each permitted lysine deprivation to induce *cat-86* Thr (data not shown).

***cat-86* is not inducible in a *relC* mutant.** Thiostrepton-resistant mutants of *B. subtilis* exhibit a relaxed phenotype (18). The relevant mutation is within a gene designated *relC*, which maps to the ribosomal protein gene cluster (9). Mutations in *relC* result in the absence of protein L11 from the large ribosomal subunit (18). It seems reasonable to believe that L11 is part of a ribosomal protein complex involved in the synthesis of ppGpp.

To test the effect of a mutation in *relC* on *cat-86* induction, strain BD54 was transformed with DNA from strain IS169 (*relC*). Thiostrepton-resistant transformants were selected, and these were fully sensitive to both chloramphenicol and amicetin, the drug inducers of *cat-86*. When we attempted to induce *cat-86* Thr in the *relC* defective derivatives of BD54,

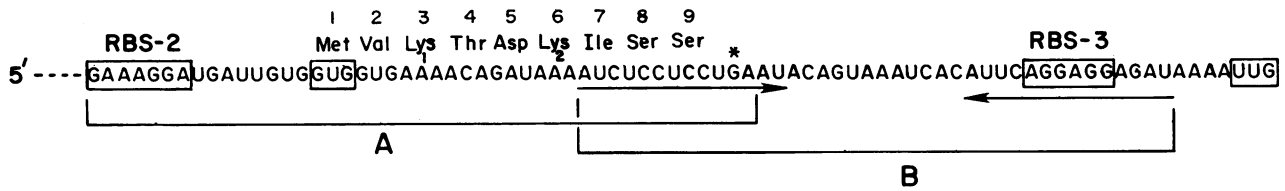

FIG. 1. Regulatory region 5′ to the *cat-86* coding sequence which is essential to inducible expression of the gene. The essential elements of the regulatory sequence are a pair of inverted repeats that sequester *cat-86* ribosome-binding site 3 (RBS-3) in RNA secondary structure (designated domain B). Domain A consists of ribosome-binding site 2 (RBS-2) and a leader of nine codons. *cat-86* induction results when a ribosome is stalled in the leader so that the aminoacyl site (A site) occupies leader codon 6 (1, 5). *cat-86* Thr is a mutant of *cat-86* in which leader codon 3 (Lys-1) is changed to a theonine codon, ACA (5).

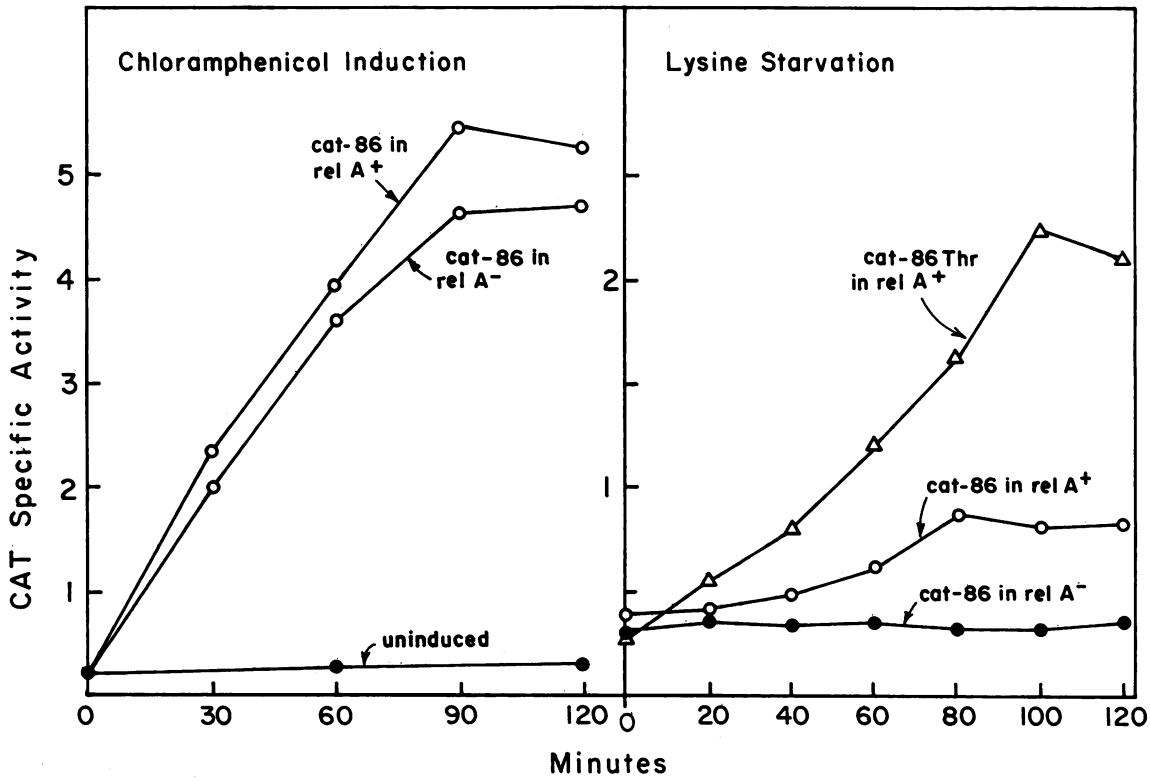

FIG. 2. Induction of *cat-86* in *relA*$^+$ and *relA* strains of *B. subtilis*. Typical chloramphenicol induction profiles of *cat-86* in BR151 (*relA*$^+$) and IS56 (*relA*) and the lack of induction of *cat-86* in IS56 (*relA*) by lysine starvation are shown. *cat-86* Thr also failed to induce by lysine starvation in IS56 and gave results identical to those shown for *cat-86*. Induction by lysine starvation of *cat-86* and *cat-86* Thr in a *relA*$^+$ host (BR151) is shown for comparison. Inductions in IS58 were comparable to those performed in BR151.

no increase in CAT specific activity was seen regardless of whether the inducing regimen used chloramphenicol or lysine starvation (Table 2).

The lack of *cat-86* induction in the *relC* mutant strain was probably not due to the relaxed phenotype of the cells, since the *relA* mutation did permit drug induction of *cat-86*. Rather, we suspect that the structural alteration of the ribosome in *relC* mutants may prevent the ribosomal function that is involved in destabilization of the RNA stem-loop (1, 18).

Basis for the lack of starvation-mediated induction of *cat-86* in a *relA* mutant. The observation that *cat-86* expression can be induced by drugs in a *relA* mutant indicated a lack of involvement of the stringent response in the regulatory mechanism. By contrast, we assumed that some aspect of the stringent response was essential for the successful induction of *cat-86* by amino acid starvation, since this failed to occur in a *relA* mutant. Successful induction of *cat-86* by amino acid starvation (i.e., lysine deprivation) requires that lysine residues become available during the starvation period to allow translation of the *cat-86* coding sequence; there are 16 lysine codons in *cat-86* (8). We previously proposed that the free lysines result from intracellular protein turnover that is triggered by the amino acid starvation regimen (5). In *E. coli*, starvation for a single amino acid has been shown to activate protein turnover, and this turnover did not occur in a *relA* mutant (20). Thus, we chose to determine whether the lack of starvation-mediated induction of *cat-86* in the *relA* strain was due to a lack of availability of lysine residues, which are essential to *cat-86* translation. To test this we grew the *relA* defective strain IS56 (*cat-86* Thr) to the mid-log phase and washed and suspended the cells in minimal

TABLE 2. Behavior of Rel$^+$ and Rel$^-$ strains with respect to *cat-86* Thr induction

Strain[a]	[^{3}H]uracil incorporation[b] (cpm)	CAT sp act during:			
		Lysine starvation		Chloramphenicol induction	
		0 min	120 min	0 min	120 min
ZA101 (*relA*$^+$)	3,000	0.9	3.6	0.8	3.1
ZA101 (*relA*)	16,000	0.8	1.0	0.8	2.5
BD54 (*relC*$^+$)	2,500	0.9	3.1	0.7	3.0
BD54 (*relC*)	13,000	0.8	0.8	0.9	0.7

[a] ZA101 strains designated *relA*$^+$ and *relA* are two Leu$^+$ transductants of ZA101 generated by using PBS1 grown on IS56. These are typical of the 38 transductants examined. The *rel* genotype is inferred from the extent of incorporation of [^{3}H]uracil during lysine deprivation.

[b] Cells were starved for lysine as described in Materials and Methods but for only 30 min. After the first 5 min of starvation [^{3}H]uracil (0.01 μCi/ml) was added. After the 30-min experimental period the cells (5 ml) were precipitated with cold trichloroacetic acid and counted for radioactivity.

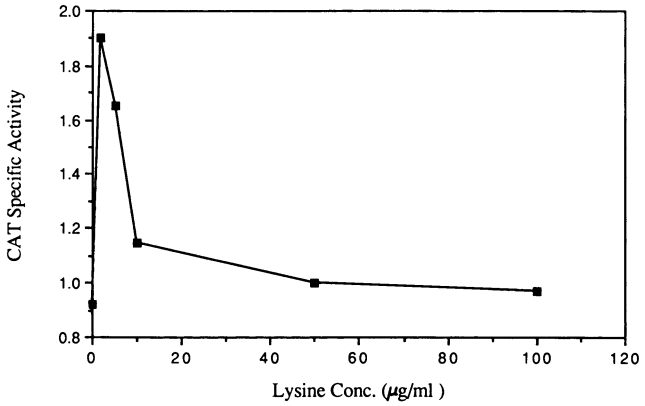

FIG. 3. Induction of *cat-86* Thr expression in a *relA* mutant in increasing concentrations of lysine. A *relA* mutant of ZA101 (*cat-86* Thr) was grown to the mid-log phase in minimal medium fully supplemented with all amino acids (5). The cells were washed and distributed among six flasks containing no lysine or lysine at a final concentration of 2, 5, 10, 50, or 100 μg/ml. Cells were shaken for 2 h at 37°C and assayed for CAT and total protein.

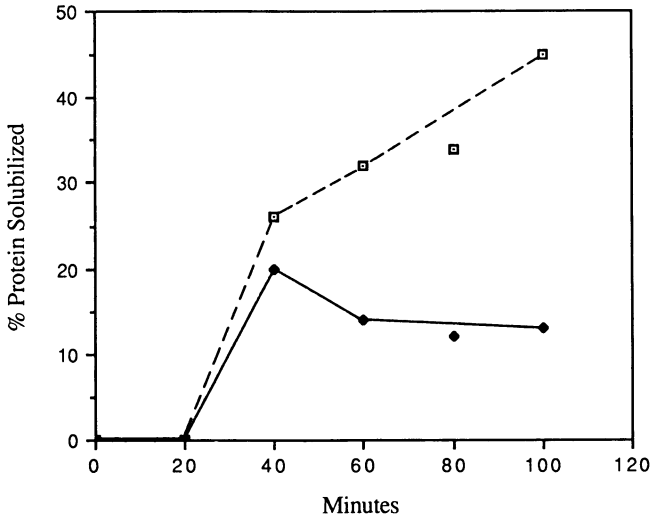

FIG. 4. Solubilization of *B. subtilis* proteins during amino acid starvation. ZA101 (*cat-86* Thr) *relA* (◆) or Rel$^+$ (⊡) was labeled for 2 h with a ^{3}H-amino acid mixture (0.5 μCi/ml). Cells were then washed and starved for lysine. Samples of 5 ml were periodically removed and precipitated with 10% trichloroacetic acid; the precipitates were collected on filters, dried, and assayed.

medium containing all amino acids except lysine. The cells were then distributed among six flasks. Lysine was added to five of the flasks at concentrations ranging from 2 to 100 μg/ml. Incubation was continued for 2 h, and CAT was then assayed. The absence of lysine from the starvation culture failed to activate *cat-86* Thr expression in the *relA* mutant host (Fig. 3), whereas starvation for lysine in a wild-type host (*relA*$^+$) activated *cat-86* expression (Fig. 2) (5). However, the addition of low levels of lysine (2 to 5 μg/ml) to the starvation culture permitted expression of *cat-86* Thr, and the addition of high levels of lysine to the starvation culture blocked induction, presumably because high lysine levels prevent ribosome stalling in the leader. Thus, we think it likely that the failure of lysine starvation to activate *cat-86* Thr expression in the *relA* mutant is due to a requirement for the stringent response in initiating intracellular protein turnover.

To directly determine whether the *relA* mutation prevented intracellular protein turnover after amino acid starvation, one each of the *relA*$^+$ and *relA* mutant transductants of ZA101 was uniformly labeled with a mixture of ^{3}H-amino acids, washed, and starved for lysine. Starvation for lysine triggered intracellular protein turnover in the *relA*$^+$ host but much less so in the *relA* host (Fig. 4). Thus, the lack of expression of *cat-86* in a *relA* host after starvation-mediated induction appears to be due to an absence of intracellular protein turnover. As shown first in *E. coli* and here in *B. subtilis*, intracellular protein turnover, which is activated by amino acid starvation, requires the *relA* gene product.

DISCUSSION

The genetic basis for the stringent response in *B. subtilis* has been studied to a limited extent, and the available information suggests that the mechanism in *B. subtilis* is probably similar to that in *E. coli* (4, 16). It is evident that the ribosome plays a central role both as a sensor for the signal that initiates the stringent response and as the site of synthesis of ppGpp. Because the *B. subtilis* ribosome also plays a central role in the induction of the translation of *cat-86* mRNA, we chose to determine whether the factors involved in the stringent response influenced *cat-86* induction.

The *relC* mutation has a pleiotrophic effect on *B. subtilis*. The mutation confers thiostrepton resistance and the relaxed phenotype, and cells carrying the mutation grow extremely slowly. Our studies of *relC* indicate that this mutation abolishes induction of *cat-86* by either chloramphenicol or amino acid starvation. Since a mutation in *relA* permits *cat-86* induction by drugs, it seems unlikely that expression of the gene requires the stringent response. We therefore presume that the structural alteration of the ribosome (18) due to the *relC* mutation interferes with the ability of the ribosome to destabilize the *cat-86* RNA stem-loop.

relA is believed to be the structural gene for stringent factor, and strains carrying a *relA* mutation failed to permit induction of *cat-86* by amino acid starvation. Our data indicate that the absence of starvation-mediated induction of *cat-86* in *relA* mutant strains is most likely because such strains fail to significantly turn over host proteins during the amino acid starvation regimen. Consequently, the amino acid that is withdrawn during the starvation is not made available by host proteolysis. Thus, *cat-86* cannot be fully translated to yield the active enzyme.

Initiation of protein turnover in *relA*$^+$ strains of *B. subtilis* was not detected until the cells had been starved for lysine for about 20 min (Fig. 4). This agrees well with induction of *cat-86* expression by lysine starvation, which also was virtually undetected until about 20 min after lysine removal (Fig. 2). We presume that this lag in both proteolysis and induction reflects the presence of intracellular pools of charged lysyl tRNAs that are not removed by the washing procedure. However, once proteolysis began the level of solubilization of proteins by the *B. subtilis relA*$^+$ cells was higher than has been reported in *E. coli* (20). *B. subtilis* produces high levels of extracellular proteases; it is conceivable that, although the initiation of proteolysis is intracellular and *relA* dependent, latter stages of the proteolysis may result from the action of the extracellular proteases on proteins that were partially degraded by intracellular and *relA*-dependent proteolytic events.

The stringent response affects a variety of processes in *E.*

coli (4), and mutations that block the stringent responses are consequently pleiotropic. The stringent response in *B. subtilis* has not yet been extensively examined, and the present study appears to represent the first demonstration of a phenotype other than RNA synthesis which is affected by the stringent response in this gram-positive cell.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI21350 from the National Institutes of Health.

We thank I. Smith for strains.

LITERATURE CITED

1. **Alexieva, Z., E. J. Duvall, N. P. Ambulos, Jr., U. J. Kim, and P. S. Lovett.** 1988. Chloramphenicol induction of *cat-86* requires ribosome stalling at a specific site in the leader. Proc. Natl. Acad. Sci. USA **85**:3057–3061.
2. **Bott, K. F., and G. A. Wilson.** 1967. Development of competence in the *Bacillus subtilis* transformation system. J. Bacteriol. **94**:562–570.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–252.
4. **Cashel, M., and K. E. Rudd.** 1987. The stringent response, p. 1410–1438. *In* F. C. Neidhardt (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
5. **Duvall, E. J., N. P. Ambulos, Jr., and P. S. Lovett.** 1987. Drug-free induction of a chloramphenicol acetyltransferase gene in *Bacillus subtilis* by stalling ribosomes in a regulatory leader. J. Bacteriol. **169**:4235–4241.
6. **Duvall, E. J., and P. S. Lovett.** 1986. Chloramphenicol induces translation of the mRNA for a chloramphenicol resistance gene in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **83**:3939–3943.
7. **Duvall, E. J., S. Mongkolsuk, U. J. Kim, P. S. Lovett, T. M. Henkin, and G. H. Chambliss.** 1985. Induction of the chloramphenicol acetyltransferase gene *cat-86* through the action of the ribosomal antibiotic amicetin: involvement of a *Bacillus subtilis* ribosomal component in *cat* induction. J. Bacteriol. **161**:665–672.
8. **Harwood, C. R., D. M. Williams, and P. S. Lovett.** 1983. Nucleotide sequence of a *Bacillus pumilus* gene specifying chloramphenicol acetyltransferase. Gene **24**:163–169.
9. **Henner, D. J., and J. A. Hoch.** 1980. The *Bacillus subtilis* chromosome. Microbiol. Rev. **44**:57–82.
10. **Lovett, P. S.** 1985. Antibiotic-inducible regulation of a plasmid gene encoding chloramphenicol acetyltransferase in *Bacillus subtilis*, p. 397–400. *In* D. Schlessinger (ed.), Microbiology—1985. American Society for Microbiology, Washington, D.C.
11. **Lovett, P. S., and K. M. Keggins.** 1979. *Bacillus subtilis* as a host for molecular cloning. Methods Enzymol. **68**:342–357.
12. **Lovett, P. S., and S. Mongkolsuk.** 1987. Promoter probe plasmids for gram-positive bacteria, p. 363–384. *In* R. L. Rodriguez and D. T. Denhardt (ed.), Vectors: a survey of molecular cloning vectors and their uses. Butterworths, Boston.
13. **Lovett, P. S., and F. E. Young.** 1970. Genetic analysis of *Bacillus pumilus* by PBS1-mediated transduction. J. Bacteriol. **101**:603–608.
14. **Price, V. L., and J. A. Gallant.** 1983. *Bacillus subtilis relG* mutant: defect in glucose uptake. J. Bacteriol. **153**:270–273.
15. **Shaw, W. V.** 1975. Chloramphenicol acetyltransferase from chloramphenicol resistant bacteria. Methods Enzymol. **43**:737–755.
16. **Smith, I.** 1982. The translational apparatus of *Bacillus subtilis*, p. 111–145. *In* D. Dubnau (ed.), The molecular biology of the bacilli, vol. 1. Academic Press, Inc., New York.
17. **Smith, I., P. Paress, K. Cabane, and E. Dubnau.** 1980. Genetics and physiology of the *rel* system of *Bacillus subtilis*. Mol. Gen. Genet. **178**:271–279.
18. **Smith, I., P. Paress, and S. Pestka.** 1978. Thiostrepton-resistant mutants exhibit relaxed synthesis of RNA. Proc. Natl. Acad. Sci. USA **75**:5993–5997.
19. **Swanton, M., and G. Edlin.** 1972. Isolation and characterization of an RNA relaxed mutant of *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **46**:583–588.
20. **Voellmy, R., and A. L. Goldberg.** 1980. Guanosine-5′-diphosphate-3′-diphosphate (ppGpp) and the regulation of protein breakdown in *Escherichia coli*. J. Biol. Chem. **255**:1008–1014.
21. **Yansura, D. G., and D. J. Henner.** 1984. Use of the *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **81**:439–443.