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Nutritional Regulation of Degradation of Aspartate Transcarbamylase and of Bulk Protein in Exponentially Growing *Bacillus subtilis* Cells

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The rate of degradation of aspartate transcarbamylase in exponentially growing *Bacillus subtilis* cells was determined by measurement of enzyme activity after the addition of uridine to repress further enzyme synthesis and by specific immunoprecipitation of the enzyme from cells grown in the presence of [^{3}H]leucine. Aspartate transcarbamylase was degraded with a half-life of about 1.5 h in cells growing on a glucose-salts medium with NH_4^+ ions as the sole source of nitrogen. Replacement of NH_4^+ in this medium with a combination of the amino acids aspartate, glutamate, isoleucine, proline, and threonine reduced the degradation rate to an undetectable level. Various other amino acids and amino acid mixtures had smaller effects on the rate of degradation. The carbon source also influenced the degradation rate, but to a smaller extent than the nitrogen source. The effects of these nutritional variables on the rate of bulk protein turnover in growing cells were generally similar to their effects on degradation of aspartate transcarbamylase. Since the degradation of aspartate transcarbamylase has been shown to be 10 to 20 times faster than bulk protein turnover, the results suggest that a substantial portion of protein turnover in growing cells represents regulatable, rapid degradation of a number of normal proteins, of which aspartate transcarbamylase is an example.

It has been shown by many investigators that protein turnover in growing bacteria is very slow (5, 10, 13). Largely on the basis of studies with *Escherichia coli*, it has been concluded that most of the proteins of growing cells are stable, whereas a small fraction of the protein turns over rapidly (8, 12–14). It has been suggested that this fraction consists largely of defective proteins, leader sequences, and processing of precursor proteins. The possibility that normal enzymes may turn over in growing bacteria as they do in mammalian cells has only recently received attention.

We were drawn to these issues by our studies of the degradation of biosynthetic enzymes in sporulating *Bacillus subtilis* cells (9, 18, 20). In particular, Maurizi et al. (9) showed that aspartate transcarbamylase is stable when the cells are growing exponentially on a glucose-nutrient broth medium, but is rapidly degraded when the cells are starved for glucose or other nutrients. However, Donachie had presented evidence in 1965 (4) that aspartate transcarbamylase was simultaneously synthesized and degraded in *B. subtilis* cells grown on a glycerol-minimal salts medium. Donachie's results led us to investigate the effects of nutritional conditions on the stability of aspartate transcarbamylase and of bulk cellular protein in exponentially growing *B. subtilis* cells. We report here that the stability of this enzyme and of a small, but significant, fraction of cellular protein is markedly regulated in growing *B. subtilis* cells by nutrients, especially amino acids, in the medium. The results indicate that the proteins degraded in growing cells include a subpopulation of the normal intracellular enzymes—of which aspartate transcarbamylase is an example—whose stability is subject to physiological regulation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The standard strain used in these studies was *B. subtilis* 168 (Trp^-), provided by L. Leon Campbell. Strains BR16 (Rel^+ Lys^- Trp^-) and BR17 (Rel^- Lys^- Trp^-) were obtained from M. Swanton (17).

The growth medium was a chemically defined mixture based on the medium of Anagnostopoulous and Spizizen (1) and supplemented with 2% of the usual level of salts added to a nutrient sporulation medium devised by Hanson et al. (6). The medium contained the following (final concentrations): 15 mM $(NH_4)_2SO_4$, 61 mM K_2HPO_4, 44 mM KH_2PO_4, 3.4 mM sodium citrate. 1.3 mM $MgSO_4$, 0.5 mM $CaCl_2$, 5 mM $FeSO_4$, 10 μM $MnCl_2$, and 6.7 mM KCl. The carbon source was 0.15% (wt/vol) glucose unless otherwise stated. When $(NH_4)_2SO_4$ was absent as the nitrogen

source, it was replaced by 15 mM K_2SO_4. Amino acid supplementation was as described below, except that tryptophan was always added at 50 μg/ml.

Five milliliters of supplemented nutrient broth (9) was inoculated with a loop of stock culture and cultivated for 7 to 10 h; a loopful of this culture was used to inoculate 5 ml of defined medium of composition identical to that to be used in the final culture and cultured overnight. This intermediate culture was transferred to the final culture, usually 500 to 750 ml, in a 2-liter Bellco flask. All liquid cultures were cultivated at 37°C with shaking at 280 rpm. Cell density was followed turbidimetrically with a Klett-Summerson colorimeter with a no. 66 filter.

Aspartate transcarbamylase assay. Aspartate transcarbamylase was assayed by the colorimetric procedure previously described (2), except that the assay buffer was 0.4 M Tris-acetate (pH 8.2), and a modified colorimetric determination of carbamylaspartate was used (15).

Immunochemical determination of aspartate transcarbamylase. The method developed by Maurizi et al. (9) was used for determination of aspartate transcarbamylase. When aspartate transcarbamylase synthesis was interrupted by uridine addition, the bacteria were cultured in 750 ml of medium containing 2 μg of leucine per ml and a total of 1 mCi of [4,5-^{3}H]leucine (New England Nuclear Corp.) for at least three generations. Uridine (100 μml) was added at a turbidity of about 25 Klett units, and 100-ml samples of culture were collected at intervals thereafter. In other experiments cells were labeled with [^{3}H]leucine and then transferred to nonradioactive medium. In these cases cells were grown in 200 ml of medium containing 2 μg of leucine per ml and a total of 1 mCi of [^{3}H]leucine. When the turbidity reached 60 Klett units, the cells were collected by centrifugation at 37°C and suspended in 750 ml of fresh medium of the same composition, except that 200 μg of nonradioactive leucine per ml replaced the [^{3}H]leucine. After 15 min of incubation to reestablish normal growth, 100-ml samples were collected at intervals.

Cells from the 100-ml samples were harvested by centrifugation, and extracts were prepared for immunoprecipitation as described by Maurizi et al. (9). Preparation of anti-aspartate transcarbamylase antibodies and immunoprecipitation were conducted as described previously (9), except that sufficiently specific antibodies were obtained without negative absorption or purification by affinity chromatography. During immunoprecipitation 20 μg of pure carrier aspartate transcarbamylase and a threefold excess over equivalence of antibody were added to each (1-ml) sample. After the immunoprecipitates were washed three times with cold 50 mM Tris-hydrochloride buffer (pH 7.9) containing 1 M NaCl, 1% Triton X-100, and 0.5 mM EDTA, they were frozen, disrupted with a Vortex mixer while thawing, and dissolved in 100 μl of 0.065 M Tris (free base), 10% glycerol, 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 4 M urea, and a trace of bromphenol blue (all at pH 6.8), heated at 100°C for 5 min, and analyzed by electrophoresis as described previously (9), except that polyacrylamide gels were prepared with 10% monomer by the method of Laemmli (7).

Determination of bulk protein turnover rates. The cells were labeled by cultivation for at least three generations in 25 ml of medium containing 40 μCi of [^{3}H]leucine (5 μCi/nmol). At a turbidity of about 60 Klett units, the cells were collected by centrifugation at room temperature, washed once with chase medium, suspended in 75 to 100 ml of chase medium, which was identical to the labeling medium except that [^{3}H]leucine was replaced with 200 μg of nonradioactive leucine per ml. After 15 min of incubation to reestablish normal growth, 1-ml samples were collected at 12-min intervals. Trichloroacetic acid-soluble and -insoluble radioactivity was determined as previously described (9), except that 0.1 ml of bovine serum albumin (10 mg/ml) was added after the trichloroacetic acid to yield better precipitation, and the final wash of the precipitate was performed with cold ethanol-diethyl ether (1:1). Turnover rates were determined from the rate of increase in trichloroacetic acid-soluble radioactivity with time, expressed as the percentage of total soluble plus insoluble radioactivity. For the period of measurement used (1.5 to 1.8 h), this rate was assumed to be zero order.

RESULTS

Stability of aspartate transcarbamylase in growing cells. The stability of aspartate transcarbamylase in growing cells could be conveniently investigated by culturing the bacteria on the medium of choice until a turbidity of about 30 Klett units was reached. Then 100 μg uridine per ml of culture was added, which quickly and completely repressed any further aspartate transcarbamylase synthesis. Cultivation was continued, and samples were withdrawn at frequent intervals for aspartate transcarbamylase assays. Turbidity was also followed to ensure that exponential growth continued and that the growth rate was not altered by uridine addition. The stability of aspartate transcarbamylase in cells growing in nutrient broth (9) could be reproduced in a chemically defined glucose-salts medium if the 20 amino acids commonly found in proteins were the sole source of nitrogen in the medium (Fig. 1A). When the cells were grown on a medium in which these amino acids were replaced with 15 mM $(NH_4)_2SO_4$ as the nitrogen source, aspartate transcarbamylase activity declined with a half-life of about 1.5 h after uridine addition (Fig. 1B).

An analysis of the effects of specific amino acids and amino acid mixtures on the stability of aspartate transcarbamylase with the technique described above led to the identification of a minimal complement of five amino acids—aspartate, glutamate, isoleucine, proline, and threonine—that would prevent inactivation of the enzyme in growing cells (Table 1). Omission of one or more of this group led to instability of aspartate transcarbamylase, but with a longer half-life than when NH_4^+ was the nitrogen source. Supplementation of the medium with the other 15 amino acids stabilized the enzyme relative to cells growing on NH_4^+, but the

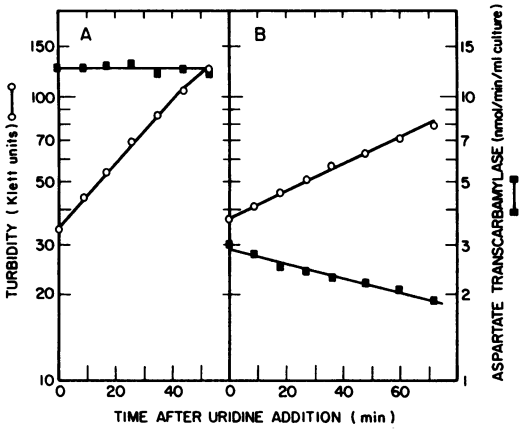

FIG. 1. Stability of aspartate transcarbamylase activity in growing *B. subtilis* cells after the addition of uridine to 100 μg/ml. A, The 20 amino acids commonly found in proteins (see footnote *d* to Table 1) were present as the nitrogen source at 100 μg each per ml. Doubling time was 26 min. B, The medium was identical to A, except that the amino acids were replaced by 15 mM $(NH_4)_2SO_4$. Doubling time was 62 min. Symbols: (■) aspartate transcarbamylase activity; (○) turbidity of the culture.

enzyme was still slowly inactivated. The addition of NH_4^+ to media containing amino acid mixtures that partially stabilized aspartate transcarbamylase during growth always increased the half-life of the enzyme further, but never yielded complete stability. These experiments, and other experiments not shown in which higher concentrations of amino acids were used showed clearly that stabilization of aspartate transcarbamylase was not simply a matter of total nitrogen content in the medium, but seemed to be mediated by the intracellular level of specific nitrogenous metabolites.

Carbon sources also affected the stability of aspartate transcarbamylase in growing cells. When the combined mixture of aspartate, glutamate, isoleucine, proline, and threonine was added to cells growing with 0.2% (wt/vol) malate, glycerol, or lactate as carbon sources, the enzyme decayed with a half-life ranging from 5 to 8 h.

The stability of aspartate transcarbamylase during exponential growth on glucose-salts medium with $(NH_4)_2SO_4$ as the nitrogen source was also examined in an isogenic stringent (BR16)

TABLE 1. Effects of amino acid supplementation on the stability of aspartate transcarbamylase activity and bulk cell protein in vivo during exponential growth

Additions to minimal medium[a]	NH_4^+	Doubling time (min)	Half-life of aspartate transcarbamylase[b] (h)	Protein degradation rate[c] (%/h)
20 amino acids[d]	−	25	>10	1.0
20 amino acids[d]	+	24	>10	–[e]
NH_4^+	+	61	1.5	3.0
Asp, Glu, Ile, Pro, Thr	−	47	>10	1.9
Asp, Glu, Ile, Pro, Thr	+	50	>10	1.6
Glu, Ile, Pro, Thr	−	47	3.5	–
Glu, Ile, Pro, Thr	+	49	3.8	–
Asp, Ile, Pro, Thr	−	49	2.9	–
Asp, Ile, Pro, Thr	+	51	6	1.8
Ile, Pro, Thr	−	78	1.7	2.4
Ile, Pro, Thr	+	53	5	–
Glu, Ile, Thr	−	52	2.0	–
Asp, Glu	−	71	1.6	3.1
Asp, Pro	−	66	1.7	–
15 amino acids[d]	−	37	3.6	2.3
15 amino acids[d]	+	35	8	–
13 amino acids[d]	−	40	2.6	2.7
13 amino acids[d]	+	44	3.7	–
Leu (200 μg/ml)	+	67	1.3	–

[a] *B. subtilis* 168 was grown on minimal medium (see text) containing in all cases 0.15% glucose, 50 μg of Trp per ml, 15 mM $(NH_4)_2SO_4$ when indicated by + in the second column, and the amino acids shown at 100 μg each per ml. Experiments on degradation of aspartate transcarbamylase and on bulk protein degradation were conducted in parallel cultures under the same conditions.

[b] Half-lives were estimated from the slopes of semilogarithmic plots of enzyme activity versus time after the addition of 100 μg of uridine per ml. Variability was about ±10% from experiment to experiment.

[c] Estimated from rates of release of [^{3}H]leucine from labeled cells as described in the text. Variability was about ±20% from experiment to experiment.

[d] Amino acid additions were as follows: for 20 amino acids, Ala, Arg, Asp, Asn, Cys, Glu, Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val; for 15 amino acids, all of the above except Asp, Glu, Ile, Pro, and Thr; for 13 amino acids, same as 15 amino acids except also lacking Asn and Gln.

[e] –, Not determined.

and relaxed (BR17) pair of strains (17). (Lysine and tryptophan were also present at 50 μg/ml to satisfy auxotrophic requirements.) Doubling times under these conditions were 49 and 60 min for strains BR16 and BR17, respectively. The half-life of the enzyme after uridine addition was considerably longer in the Rel^- strain (about 6 h) than its Rel^+ sibling (2.4 h). This observation suggests that effects of nitrogen sources on aspartate transcarbamylase degradation may be mediated via the Rel system, but further experiments would be required to establish this suggestion firmly.

Immunochemical evaluation of the stability of aspartate transcarbamylase in growing cells. The preceding experiments suggest that aspartate transcarbamylase is simultaneously synthesized and degraded in *B. subtilis* cells growing on a glucose–NH_4^+–salts medium, and that the rate of degradation is regulated by amino acid supplementation of the medium. The experimental technique employed does not, however, distinguish between inactivation (without subsequent degradation) and degradation of the enzyme. Furthermore, it is not certain that uridine addition, which was used to block aspartate transcarbamylase synthesis, does not also alter the rate of degradation of the enzyme. These issues could be resolved by following the fate of aspartate transcarbamylase protein in growing cells labeled with [^{3}H]leucine by specific immunoprecipitation, as has been carefully developed by Maurizi et al. (9).

Two kinds of experiments were performed. The first was very similar to those already described in which uridine was added to block subsequent aspartate transcarbamylase synthesis, except in this case the fate of the enzyme protein was followed immunochemically. It has been previously demonstrated that loss of cross-reactive material in this assay results from degradation in vivo (9). The two culture conditions differing most in the stability of aspartate transcarbamylase were examined, namely, NH_4^+ versus 20 amino acids as the sole nitrogen sources. As shown above, both aspartate transcarbamylase activity and cross-reactive protein were stable in cells growing on amino acids after uridine addition. After the addition of uridine to cells growing on NH_4^+, aspartate transcarbamylase activity declined with a half-life of 1.7 h, and the cross-reactive protein decreased with essentially the same half-life, 1.9 h. These results show that inactivation of aspartate transcarbamylase is accompanied by simultaneous degradation, as in the case in stationary-phase cells (9). No inactive cross-reactive protein was accumulated.

The second kind of immunochemical experiment involved growth of the cells in a medium containing [^{3}H]leucine until the cells accumulated an easily detectable amount of radioactive aspartate transcarbamylase. The cells were then harvested by centrifugation at 37°C and suspended in an identical medium containing an excess of nonradioactive leucine. (It is shown in Table 1 that the addition of a high concentration of leucine does not alter the stability of aspartate transcarbamylase in growing cells.) At various times after transfer, samples were harvested for assay of activity and for specific immunoprecipitation of aspartate transcarbamylase. Again, the experiment was conducted in media supplemented with NH_4^+ or with 20 amino acids as the nitrogen source. When the cells grew on amino acid-supplemented medium, the aspartate transcarbamylase synthesized during the labeling period was completely stable after transfer to a nonradioactive medium (until the cells entered the stationary phase) (Fig. 2A). When the cells were grown with NH_4^+ as the nitrogen source, however, the labeled enzyme clearly decayed after transfer with a half-life of about 2 h (Fig. 2B). In both cases growth and aspartate transcarbamylase synthesis continued at normal rates after the transfer. The results of Fig. 2B establish conclusively that simultaneous synthesis and degradation of aspartate transcarbamylase occurs during exponential growth of *B. subtilis* on a medium lacking amino acids. Furthermore, the similarity in the rates of decay of the cross-

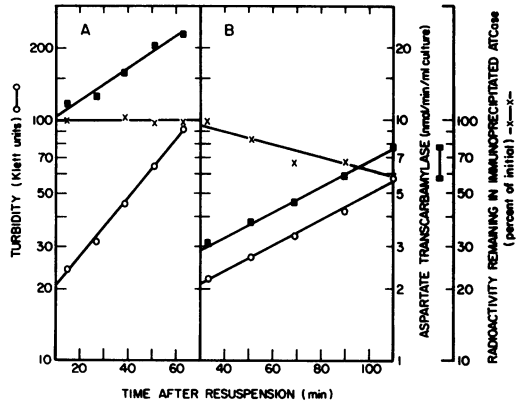

FIG. 2. Immunochemical determination of the stability of aspartate transcarbamylase (ATCase) after transfer to a medium containing excess nonradioactive leucine. Culture turbidity (○), aspartate transcarbamylase activity (■), and the amount of radiolabeled immunoprecipitable protein corresponding to aspartate transcarbamylase protein (X) were determined as described in the text. The media used were identical, except the nitrogen source in A was twenty amino acids (footnote *d*, Table 1) at 100 μg each per ml, and the nitrogen source in B was 15 mM $(NH_4)_2SO_4$. Doubling times were the same as in Fig. 1. The initial radioactivity in the immunoprecipitate in A was 6,450 cpm; in B, it was 1,910 cpm.

reactive material whether or not uridine was added indicates that intracellular pyrimidines play little or no role in regulating the degradation of aspartate transcarbamylase in growing cells.

Stability of bulk protein in growing cells. The foregoing results with aspartate transcarbamylase, which constitutes less than 0.2% of the total cell protein (2), led us to inquire whether the stability of other proteins might be regulated in growing cells in a similar manner. This could be examined for bulk cellular proteins by measuring the release of [^{3}H]leucine from labeled cells to a trichloroacetic acid-soluble fraction after a transfer to a medium containing an excess of nonradioactive leucine, as has been done by many other laboratories (5, 10, 13, 14). Bulk protein degradation rates estimated in this way for *B. subtilis* cells growing exponentially in various media are given in Table 1. In all cases, media were used for which the stability of aspartate transcarbamylase under the same conditions was known. Protein degradation rates ranged from a minimum of 1.0%/h for cells growing with 20 amino acids to a maximum of about 3%/h observed with the simple glucose–NH_4^+–salts medium. Intermediate degradation rates were obtained when the minimal medium was supplemented with various amino acid mixtures. In fact, there was a good general correlation between the stability of aspartate transcarbamylase and the rate of bulk protein degradation between the values of 2 to 3%/h (Fig. 3). While we attach no theoretical interpretation to it, this correlation suggests to us that aspartate transcarbamylase is a member of a family of proteins whose stability is regulated by nitrogen nutrition in similar ways.

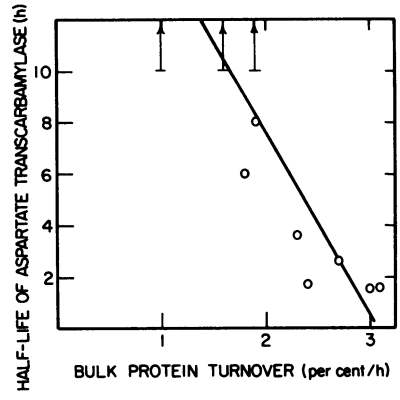

FIG. 3. Correlation between the half-life of aspartate transcarbamylase after repression by uridine and the rate of bulk protein turnover on the same medium. Data were taken from Table 1. Instances in which the half-life of aspartate transcarbamylase was ≥10 h are shown with vertical arrows.

DISCUSSION

The experiments in this paper establish that under some conditions aspartate transcarbamylase is simultaneously synthesized and degraded in exponentially growing *B. subtilis* cells. Previous studies have established that simultaneous synthesis and degradation occur for a small fraction of bulk cell protein in bacteria. Analysis of this process by labeling and separation of individual proteins by two-dimensional electrophoresis and isoelectric focusing indicated that in *E. coli* only a fraction of the proteins turn over in this way, whereas the majority of proteins are stable (8, 12). These and other studies have not identified the biochemical functions of the particular proteins subject to turnover in growing bacteria. Aspartate transcarbamylase is now identified as a member of this group of proteins in *B. subtilis*. Others have not been identified, but they may include other enzymes catalyzing reactions at appropriate regulatory points or possibly other enzymes of pyrimidine biosynthesis. In this context it is interesting that glutamine phosphoribosylpyrophosphate amidotransferase, the first enzyme of purine nucleotide biosynthesis, which is also degraded in stationary *B. subtilis* cells, is stable in growing cells under conditions in which aspartate transcarbamylase is turned over (M. E. Ruppen, D. A. Bernlohr, and R. L. Switzer, unpublished experiments).

Turnover of aspartate transcarbamylase in growing cells reflects neither protein processing nor degradation of an abnormal protein. The possibility that the frequency of synthesis of defective aspartate transcarbamylase molecules is sharply increased in cells growing on NH_4^+, as opposed to certain amino acid mixtures, seems very unlikely. It is not likely that the intracellular amino acid pools would be so drastically altered by growth on NH_4^+ as to account for synthesis of so much defective protein. Furthermore, such a defect would not be so selective as to affect the turnover of aspartate transcarbamylase dramatically while having only minor effects on the turnover of bulk cellular protein. It is likely that some of the protein degradation seen in cells growing in fully supplemented medium results from processing and degradation of defective proteins. The portion of bulk protein degradation that did not respond to amino acid supplementation of the medium (1 to 2%/h) may represent such degradation. We suggest that the portion of protein degradation that does respond to amino acid nutrition represents the regulatable degradation of a small family of normal proteins. Another possibility is that the amino acids in the medium bring about relatively small changes in the slow turnover of a large number of proteins. Aspartate transcarbamylase does not fit this pattern, however, because the measured degradation rate for this enzyme is 10

to 20 times that of bulk protein. The results of Larrabee et al. (8) and of Mosteller et al. (12) on the turnover of individual proteins in *E. coli* also pointed to the existence of a fraction of rapidly turning over proteins among a majority of stable proteins. The use of techniques employed by these authors would be required to establish definitively the fraction of *B. subtilis* proteins whose stability responds to nutritional factors.

The stability of aspartate transcarbamylase was markedly affected by both nitrogen and carbon sources in the medium. The presence or absence of the end product of the enzyme, i.e., pyrimidines, in the medium had little or no effect on degradation of the enzyme. There was a rough correlation among growth rate, protein degradation rate, and the rate of aspartate transcarbamylase degradation. Proteins were generally more stable in rapidly growing cells. Close examination of the data suggests that other factors were also important. Half-lives for aspartate transcarbamylase ranging from 2 h to ≥10 h were observed in cells doubling every 50 ± 5 min in various media. Our studies give little insight into the biochemical basis for the regulation of the degradative process. In particular, it is remarkable that certain amino acids seem to dominate this regulation. Yet the observed effects are too complex to be accounted for by suggesting that the stability of aspartate transcarbamylase is regulated by the intracellular pool of only one or two amino acids. The results are strongly reminiscent of the findings of May and Elliot (11), who showed that isoleucine, proline, aspartate, and glutamate were the most effective in repressing the synthesis of extracellular protease in *B. subtilis*, and those of Chaloupka and Křečková (3), who identified isoleucine and threonine as effective in the repression of extracellular protease in *B. megaterium*. Indeed, our studies were much aided by our awareness of these earlier findings. Do the amino acids act by repressing synthesis of an intracellular protease? The studies of Maurizi et al. (9) on the effects of chloramphenicol and rifampin on the degradation of aspartate transcarbamylase argue against this suggestion, because these antibiotics actually initiate degradation in cells growing on nutrient broth-glucose medium. The amino acids could serve to inhibit a protease, however. Our findings with the Rel$^-$ mutant provide a hint that the effects of amino acids, and possibly carbon sources as well, could be channeled through the Rel system via effects on levels of guanosine tetra- and pentaphosphate. There is strong evidence from Goldberg's laboratory (16, 19) that intracellular levels of these compounds are correlated with the rate of bulk protein turnover in *E. coli* during nutrient limitation. Little is known about the control of the amounts of guanosine tetra- and pentaphosphate in growing *B. subtilis* cells, however, so this suggestion requires further investigation.

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