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Energy and Calcium Ion Dependence of Proteolysis during Sporulation of *Bacillus subtilis* Cells

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Bacterial cells degrade intracellular proteins at elevated rates during starvation and can selectively degrade proteins by energy-dependent processes. Sporulating bacteria can degrade protein with apparent first-order rate constants of over 0.20 h^{-1}. We have shown, with an optimized [^{14}C]leucine-labeling and chasing procedure, in a chemically defined sporulation medium, that intracellular protein degradation in sporulating cells of *Bacillus subtilis* 168 (*trpC2*) is apparently energy dependent. Sodium arsenate, sodium azide, carbonyl cyanide *m*-chlorophenylhydrozone, and *N,N'*-dicyclohexylcarbodiimide, at levels which did not induce appreciable lysis (≤10%) over 10-h periods of sporulation, inhibited intracellular proteolysis by 13 to 93%. Exponentially growing cells acquired arsenate resistance. In contrast to earlier reports, we found that chloramphenicol (100 μg/ml) strongly inhibited proteolysis (68%) even when added 6 h into the sporulation process. Restricting the calcium ion concentration (<2 μM) in the medium had no effect on rates or extent of vegetative growth, strongly inhibited sporulation (98%), and inhibited rates of proteolysis by 60% or more. Inhibitors of energy metabolism, at the same levels which inhibited proteolysis, did not affect the rate or degree of uptake of Ca^{2+} by cells, which suggested that the Ca^{2+} and metabolic energy requirements of proteolysis were independent. Restricting the Ca^{2+} concentration in the medium reduced by threefold the specific activity in cells of the major intracellular serine proteinase after 12 h of sporulation. Finally, cells of a mutant of *B. subtilis* bearing an insertionally inactivated gene for the Ca^{2+}-dependent intracellular proteinase-1 degraded protein in chemically defined sporulation medium at a rate indistinguishable from that of the wild-type cells for periods of 8 h.

Intracellular protein degradation in growing, starving, and sporulating bacterial cells is now well documented (12, 23, 25, 27, 30, 39). In *Escherichia coli* cells, Mandelstam (22) found that degradation of normal bulk protein depended on metabolic energy; addition of sodium azide or 2,4-dinitrophenol to cultures of nongrowing cells strongly inhibited their ability to degrade intracellular protein. The studies of Olden and Goldberg (32) and St. John and Goldberg (46) extended these observations to suggest that ATP, or some other high-energy phosphate, was probably essential for the degradation of both abnormal (canavanine-containing) and normal bulk protein in nongrowing cells. During starvation, *E. coli* cells can degrade protein at rates of 4 to 5% h^{-1} and can degrade more than half their total cellular protein, an ability which presumably confers survival advantages on the cells (12, 27, 35). Degradation of bulk protein during starvation is apparently a first-order process and yields a first-order rate constant of about 0.04 h^{-1} (22; our calculation).

In growing *Bacillus subtilis* cells (4), rates of bulk protein degradation (1 to 3% h^{-1}, depending on nutritional conditions) are similar to those seen in the enteric bacteria. In contrast, during the developmental process of spore formation, cells of the species *Bacillus* have been measured to degrade intracellular protein with first-order rate constants of 0.070 to 0.27 h^{-1} (2, 40), depending on the experimental conditions. Not only is protein degradation relatively rapid during this period; it is also very extensive (40). Bernlohr (2) estimated that *Bacillus licheniformis* cells on average turn over protein two or more times during sporulation. Furthermore, because bulk protein synthesized during sporulation appeared to be degraded as rapidly as preexisting (vegetative) protein, Spudich and Kornberg (45) and Mitani and Kadota (28) have suggested that protein degradation is in some respects nonselective. Our own observations support this suggestion (40).

In the case of *B. subtilis* cells, Waindle and Switzer have established the requirement of metabolic energy for the rapid inactivation and degradation of aspartyl transcarbamylase at the onset of stationary growth (49), but the two processes were so tightly linked that Maurizi and co-workers (24) were not able to determine whether inactivation or degradation was the energy-dependent step. In addition, the degradation (but not the inactivation) of glutamine phosphoribosyl amidotransferase was inhibited if energy metabolism was severely blocked (38). On the other hand, degradation of ornithine transcarbamylase (31) and carbamyl phosphate synthetase P did not appear to require metabolic energy (33).

In contrast to the many studies with *E. coli* cells, few studies have been reported on the energy dependence of the more rapid and extensive proteolysis which occurs during spore development in *B. subtilis* cells. In the present work, we show that rates of proteolysis in cells of *B. subtilis* sporulating in a chemically defined medium are sharply reduced in the presence of inhibitors of energy metabolism and of chloramphenicol. Rates of proteolysis and total intracellular proteinase activity were both reduced by limiting calcium ion concentrations in the medium. Since the metabolic poisons used did not restrict the uptake of calcium ions, we believe that the requirements for calcium ions and ATP or energized membranes are independent requirements for optimal proteolysis. Cells of a strain containing an insertionally inactivated intracellular serine proteinase-1 (ISP-1) degraded intracellular protein at the same rates as those of the parental strain over an extended period.

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MATERIALS AND METHODS

Chemicals. Cell culturing materials were purchased from Difco Laboratories, Detroit, Mich. All inorganic salts used in growth media were from Malinckrodt (St. Louis, Mo.).

Organic and biochemical reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.) or Aldrich (Milwaukee, Wis.), except that 1,2-bis(2-amino-5-bromophenoxy) ethane *N*,*N*,*N'*,*N'*-tetraacetate, tetrapotassium salt (cell impermeant; dibromo-BAPTA) was obtained from Molecular Probes (Junction City, Ore.). The L-[U-^{14}C]leucine (specific activity, 348.3 mCi/mmol in a total of 2.5 ml) was purchased from Du Pont-NEN Research Chemicals (Boston, Mass.). Complete counting cocktail (3a70B) was obtained from Research Products, Inc. (Mt. Prospect, Ill.).

Bacterial strains and culturing methods. Studies were conducted with *B. subtilis* 168 (*trpC2*) and with *B. subtilis* 3036 (ISP$^-$) and its parent *B. subtilis* 168I, which were gifts from Mark Ruppen of Genencor (1). Strains were checked for auxotrophy as described by Geele et al. (11). Stock cultures were maintained on gelatin agar plates.

Cells were grown in the chemically defined sporulation medium (CDSM) described by Hageman et al. (14). For all experiments, 50 ml of CDSM in a 300-ml baffled sidearm flask (Bellco Glass Co., Vineland, N.J.) was inoculated with a loopful of colonies from a stock plate and incubated at 37°C with shaking (220 rpm) for 9 to 12 h in a New Brunswick G-25 rotary shaker-incubator. The growth of the culture was monitored turbidometrically with a Klett-Summerson colorimeter with a no. 66 filter; this inoculum was used when the turbidity of the culture reached 50 to 200 Klett units.

Protein degradation measurements. In experiments involving protein degradation, a 2 to 10% (vol/vol) inoculum was transferred into 50 ml of fresh medium in a 300-ml baffled sidearm flask, and growth was continued under the same conditions, with 10 μCi of L-[U-^{14}C]leucine being added to the culture 1 h after inoculation. Cells were grown to a turbidity of 220 to 240 Klett units and harvested by centrifugation for 30 s at 8,000 rpm (10,400 × *g*) in a Sorvall RC-2B refrigerated centrifuge in a GSA rotor warmed to 37°C. The supernatant fraction was decanted, and the cells were washed one time in 200 to 250 ml of fresh, warm CDSM. Washed cells were resuspended in 30 to 50 ml of warm CDSM, and the experimental flasks containing 75 ml of fresh CDSM supplemented with 2.0 mg of unlabeled L-leucine per ml were inoculated with 5 to 10% (vol/vol) of the suspension. The growth of these cultures was again monitored turbidometrically until they reached the end of exponential growth (t_0).

The methods used to measure protein degradation were a modification of those used by Maurizi et al. (24). At t_0, duplicate 1.0-ml samples were removed from each experimental flask and placed in a 1.5-ml microfuge tube containing 0.25 ml of 50% trichloroacetic acid (TCA), mixed, and spun at 12,000 rpm (9,000 × *g*) for 5 min in a Beckman Microfuge B. The supernatant fraction was decanted, and the pellet was washed twice with 5% TCA supplemented with 10 mg of L-leucine per ml. The washed pellet was then suspended in 1.0 ml of 0.2 M Tris hydrochloride–1 mM $CaCl_2 \cdot 2H_2O$ (pH 7.8) buffer, to which 0.1 ml of 10-mg/ml pronase was then added. The microfuge tubes were then incubated in a water bath at 37°C until the pellets were digested (3 to 12 h), i.e., formed a clear solution. This sample served as the reference point for the total amount of label incorporated into cells at the onset of sporulation and is designated by C_0 in plots below.

Cell samples were routinely removed at various times after t_0, added to a microfuge tube containing 0.25 ml of 50% TCA, and centrifuged at 12,000 rpm (9,000 × *g*) for 5 min. For samples taken after t_0, the TCA-soluble fraction was saved and counted to measure the L-[^{14}C]leucine released during protein breakdown (designated by C_t in the plots below).

Samples (generally, 0.1 ml of the digested pellet and 0.2 to 0.3 ml of the TCA-soluble fractions) from duplicate cultures were counted in quadruplicate in 10 ml of 3a70B complete counting cocktail for 5 min in a Packard Tri-Carb 460C scintillation counter. The disintegrations per minute values were analyzed by the statistical treatment of Shoemaker and Garland (41) for the rejection of discordant data. The standard deviation of the average value was never greater than 8% of the mean in the pellet samples or 4% of the mean in the TCA-soluble fractions. The pellets had a higher standard deviation because it was more difficult to digest the pellet fractions to complete homogeneity, whereas the supernatant fractions were already in solution.

The procedure used here makes two implicit assumptions: (i) little proteolytic activity occurs in *B. subtilis* cells during exponential growth; thus, cells at t_0 contain most of the proteins present in vegetative cells; and (ii) during sporulation, labeled leucine released intracellularly as a result of proteolysis can exit from the cell and be diluted by the exterior pool of unlabeled leucine.

We have assumed that proteolysis is a first-order process (5), and we have estimated first-order rate constants for proteolysis from plots of the relationship $\ln\{C_0/[C_0 - C_{t(\mathrm{corr})}]\} = k_1 t$, where C_0 is disintegrations per minute per milliliter of culture in the pellet at t_0, $C_{t(\mathrm{corr})}$ is the disintegrations per minute per milliliter of cell culture at times after t_0 which have been corrected for cell lysis, if it occurs, t is time in hours, and k_1 is the apparent first-order rate constant.

To correct for cell lysis, we have made the conservative postulate that any cell lysis will falsely elevate the apparent rate of degradation because of the probable degradation of released protein by extracellular proteases. To correct for lysis, we applied the following relationship: $C_{t(\mathrm{corr})}$ = dpm in medium at t_x − {dpm in pellet at t_3 × [1 − (culture turbidity at t_x/culture turbidity at t_3)]}, where dpm in pellet at t_3 is calculated by subtracting the counts released at t_3 (when cell turbidity is generally highest) from the counts in the pellet fraction measured at t_0 and t_x is time (in hours) after t_0. Typically, corrected counts were 5 to 10% lower than the actual counts, but in several cases, at the latest times sampled, corrections of 25% were made.

Azocaseinase assay. Intracellular protease activity was measured by the method of Geele et al. (11).

Protein assays. Protein concentrations were estimated by the methods described earlier (5, 11).

Measurement of calcium ion concentrations. Since a number of experiments were performed that depended on the removal of calcium from the growth medium and labware, it was necessary to routinely check for calcium in all stock solutions and the medium itself. All stock solutions were made with distilled, deionized water and were stored in plastic containers that were rinsed first with 20 mM EDTA (or EGTA [ethylene glycol tetraacetic acid], pH 7.5 to 7.8) and then several times with the distilled, deionized water. All glassware in contact with the medium or the stock solutions was soaked for 10 h in the EDTA (or EGTA) wash and rinsed three to five times with distilled, deionized water. The Ca^{2+} concentrations in all stock solutions used to prepare CDSM as well as in the final CDSM were measured

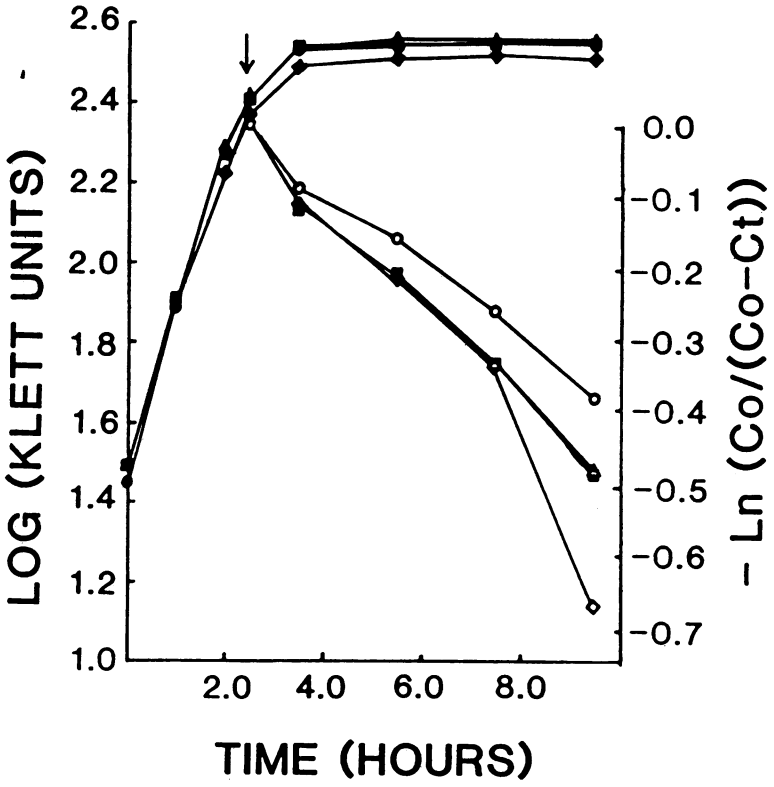

FIG. 1. Effect of varying levels of L-leucine chase on proteolysis in *B. subtilis* cells grown in CDSM. Cells were grown and samples were removed as described in Materials and Methods. The end of the exponential growth phase (t_0) is designated by the arrow. Duplicate samples were removed from duplicate flasks at t_0, t_1, t_3, t_5, and t_7, and radioactivity was counted as described in Materials and Methods. Symbols: ●, growth of cells in CDSM chased with 250 μg of L-leucine per ml; ■, growth of cells in CDSM chased with 750 μg of L-leucine per ml; ▲, growth of cells in CDSM chased with 2,250 μg of L-leucine per ml; ◆, growth of cells in CDSM chased with 6,250 μg of L-leucine per ml; ○, degradation of protein by cells chased with 250 μg of L-leucine per ml; □, degradation of protein by cells chased with 750 μg of L-leucine per ml; △, degradation of protein by cells chased with 2,250 μg of L-leucine per ml; ◇, degradation of protein by cells chased with 6,250 μg of L-leucine per ml.

with an Instrumentation Laboratory Atomic Absorption Spectrophotometer 457 and standards of 10^{-5} to 10^{-7} M $CaCl_2 \cdot 2H_2O$ solutions. Stock solutions of glutamate (1 M, pH 7.0) contained the highest levels of Ca^{2+} (9×10^{-6} to 11×10^{-6} M) of any of the stocks, and final concentrations of Ca^{2+} in CDSM itself were always less than 2 μM (near the limit of detection).

Measuring heat-stable spores. Duplicate 1.0-ml samples of sporulating cultures were removed and mixed vigorously with 10 ml of Spizizen minimal salts (44), which was then heated to 70 to 75°C for 30 min. Appropriate dilutions were plated onto gelatin plates, and colonies were counted after growth at 37°C for 48 h.

RESULTS

Effect of L-leucine concentrations on protein degradation. The primary objective of the research undertaken here was to determine whether the very rapid and extensive degradation of protein (over 80% degraded in an 8-h period) which occurs during sporulation in *B. subtilis* cells (5, 40) is an energy- or Ca^{2+}-dependent process. We chose to study this process in CDSM (14), with [^{14}C]leucine used to label cellular protein. Leucine is a suitable amino acid for studying protein degradation in *B. subtilis* cells because, in nutrient broth, exogenous leucine can exchange with internal pools of leucine, is incorporated into protein only as leucine, and is not metabolized in a detectable manner until late in spore formation (40).

Figure 1 shows the effect of the concentration of the leucine chase on degradation rates of intracellular proteins of *B. subtilis* sporulating in CDSM. Because a chase with 250 μg of L-leucine per ml resulted in a slightly lower rate of degradation than higher levels and a chase with 6,250 μg/ml interfered with both growth and sporulation compared with all other levels of treatment, a chase with 2,000 μg of L-leucine per ml was used in all subsequent experiments. Cultures containing 2.25 mg of L-leucine per ml sporulated to a level of 90% refractile bodies, similar to what was reported previously (14). Other aspects of the use of leucine as a labeling and chasing probe have been discussed elsewhere (40).

Effects of energy inhibitors on protein degradation in sporulating cells of *B. subtilis*. The action of a number of metabolic poisons on the proton motive force and ATP pools in cells of *B. subtilis* SR22, a strain derived from *B. subtilis* 168, has been thoroughly studied by Jolliffe et al. (19). We have studied the effect of sodium arsenate, an uncoupler of substrate-level oxidative phosphorylation, and three other inhibitors of energy metabolism described by Jolliffe and colleagues. The effects of these inhibitors on culture turbidities and rates of proteolysis were determined under various experimental conditions.

Initially, we tested the effects of arsenate (1 to 16 mM final concentration in the culture broths) on the growth of *B. subtilis* cells; concentrations above 8 mM caused extensive cell lysis, and levels of 2 mM or below caused no inhibition

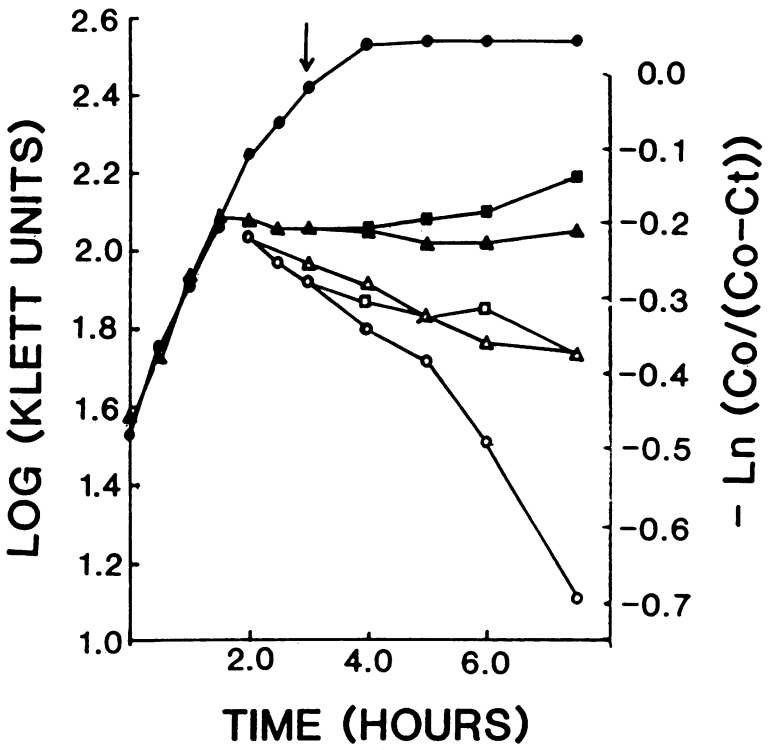

FIG. 2. Effect of 4 and 8 mM arsenate on growth and proteolysis in *B. subtilis* cells grown in CDSM. The experiment was conducted as in Fig. 1 except the first (reference) pellet sample was removed at mid-exponential growth phase (at a turbidity of 110 Klett units), and arsenate was added immediately after the first sampling. Subsequent samples were removed at t_{-1}, $t_{-0.5}$, t_0, t_2, t_3, and $t_{4.5}$, and the radioactivity in the TCA-soluble supernatant fractions was counted. Symbols: ●, growth of control cells in CDSM; ■, growth of cells to which 4 mM arsenate was added; ▲, growth of cells to which 8 mM arsenate was added; ○, degradation of protein by control cells in CDSM; □, degradation of protein by cells to which 4 mM arsenate was added; △, degradation of protein by cells to which 8 mM arsenate was added.

of growth. When 4 or 8 mM arsenate was added to cultures of cells at the mid-log phase of growth, the growth of the cells was sharply arrested (Fig. 2); arsenate inhibited the degradation of protein which occurs at both the late exponential and early stationary phases as well as the more vigorous process which occurs during early sporulation phases. The first stage of protein degradation (Fig. 2) in the untreated cells had a first-order rate constant of 0.05 h^{-1}, slightly higher than the value of 0.033 h^{-1} reported in earlier studies (4, 5). In subsequent experiments (Table 1), inhibitors were added after the end of exponential growth to minimize the chance that the energy poisons were merely blocking the entry of cells into sporulation. An unexpected feature seen with arsenate but with none of the other inhibitors was the acquisition of resistance to arsenate, which was seen clearly at 8 h (Fig. 2). In fact, when cells which had begun to grow again after being in the presence of 4 mM arsenate for several hours were transferred to fresh medium containing either no arsenate or 4 mM arsenate, they grew at the same rates (doubling times of 50 to 60 min). The acquisition of arsenate resistance by *E. coli* and *Staphylococcus aureus* cells has been observed previously and attributed to an inducible plasmid-borne efflux system (43); to our knowledge, arsenate resistance has not been reported previously for sporeforming bacteria.

In a manner similar to that used for arsenate, we systematically examined the effects of sodium azide, which conducts H^+ and inhibits cytochrome oxidase and ATPase; carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which conducts H^+, uncouples electron transport, and inhibits Ca^{2+} uptake in membrane vesicles (7); and *N,N'*-dicyclohexylcarbodiimide (DCCD), which depletes H^+ gradients and inhibits ATPase, on rates of proteolysis during the sporulation stages in *B. subtilis* cells. Initial range-finding experiments determined what levels could be used without causing appreciable lysis. Other metabolic inhibitors were found to block proteolysis, but these also decreased cell viability and were not studied further.

The results of these experiments are summarized in Table

TABLE 1. Effects of energy metabolism inhibitors on the rates of proteolysis in *B. subtilis* cells grown in CDSM

Inhibitor	Final concn	Time of addition	Period of analysis[a]	% Inhibition of k_1[b]
Arsenate	4 mM	$t_{-1.5}$	$t_1 - t_{4.5}$	80
	8 mM	$t_{-1.5}$	$t_1 - t_{4.5}$	74
	4 mM	t_0	$t_{2.25} - t_{4.25}$	62
Sodium azide	25 μM	t_1	$t_6 - t_8$[c]	14
	50 μM	t_1	$t_6 - t_8$[c]	35
	400 μM	t_3	$t_4 - t_8$	27
	800 μM	t_3	$t_4 - t_8$	27
CCCP	5 μM	t_1	$t_6 - t_8$[c]	45
	10 μM	t_1	$t_6 - t_8$[c]	93
	10 μM	t_3	$t_4 - t_8$	80
	25 μM	t_3	$t_4 - t_8$	63
DCCD	100 μM	t_1	$t_3 - t_7$	70

[a] Slope of linear portion of plot of time versus $-\ln(C_o/[C_o - C_t])$ used to calculate apparent first-order rate constant k_1.

[b] Compared with the k_1 of control rates from experiments run at the same time. All rates were corrected for lysis, if it occurred, as described in Materials and Methods.

[c] Slopes for determining k_1 were based on two sets of points.

1. Azide had the least effect, and its effects on proteolysis were not apparent until 2.5 to 3 h after its addition to cultures. CCCP was the most potent of the inhibitors tested, and its effects at both concentrations were apparent after only 1 h in the cultures; furthermore, 25 μM CCCP induced only about 13% cell lysis even after 8 h in the cultures. With DCCD, marked inhibition of proteolysis was evident after 2 h in the culture (the earliest time sampled). Even though the reduction of rates of proteolysis was evident in 1 to 3 h after addition of these inhibitors to the cultures, Table 1 compares only linear regions of the proteolysis curve in order to report comparable apparent values of the first-order rate constant, K_1. These results suggest that proteolysis in sporulating cells of *B. subtilis* has a direct or indirect requirement for either ATP or some form of energized membrane or both.

Effect of chloramphenicol on rates of proteolysis. Spudich and Kornberg (45) and Mitani and Kadota (28) have reported that when chloramphenicol (100 μg/ml) was added before t_3, it inhibited proteolysis of bulk protein, but when it was added at later times, it had a stimulatory effect on proteolysis. However, the former authors (45) show data which indicate that extensive cell lysis was induced by chloramphenicol, especially in older cultures, but make no mention of correcting for it. We have carried out a series of experiments similar to those in Table 1. Chloramphenicol (100 μg/ml) was added to cultures growing in CDSM at t_3, $t_{4.5}$, and t_6, and subsequent rates of degradation (corrected for lysis as described above) were measured between t_6 and t_8. Apparent first-order rate constants determined between t_6 and t_{10} were found to be inhibited compared with controls by 96, 90, and 68%, respectively. When added at t_3, chloramphenicol reduced proteolysis by 44% at t_5 (the earliest time sampled). Chloramphenicol (at 50 μg/ml) had a stimulatory effect on the ATP pool and no appreciable effect on the proton motive force of vegetative cells of *B. subtilis* SR22 cells (19). We believe that Spudich and Kornberg (45) considerably overestimated the rates of proteolysis when chloramphenicol was added at later times because of the extensive lysis of the cells which occurred under the conditions they used. These results also raise the possibility that the energy inhibitors could act by inhibiting protein synthesis.

Effect of calcium ions on sporulation, rates of proteolysis, and proteinase activity in *B. subtilis* cells. Because of the requirement for Ca^{2+} by bacilli for sporulation (13) and the catalytic or stabilizing effect of Ca^{2+} on the (major) ISP-1 of *B. subtilis* (29, 36), we studied the effect of Ca^{2+} on sporulation, proteinase activity, and proteolysis in *B. subtilis* cells. In CDSM, cells were strongly dependent on the addition of calcium ions to the medium to achieve maximal sporulation (Table 2). In addition, we found that although cells required no Ca^{2+} (<2 μM) for growth, they were unable to degrade protein in a normal manner when the levels were restricted in the medium (Fig. 3). In the absence of added Ca^{2+}, the apparent first-order rate constant was inhibited by 60% compared with that of cells growing in the control medium, and the rate constant was inhibited by 81% when the Ca^{2+} concentration was restricted further by addition of the selective cell-impermeant chelator dibromo-BAPTA. The levels of chelator used (200 μM) were calculated to be sufficient to chelate all the exogenous Ca^{2+}, Fe^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} added to the CDSM; no effects of the chelator on cell lysis or culture turbidity were noted. We have also consistently noted that cells appear to undergo less lysis during sporulation when grown in the absence of added calcium ions than in their presence.

TABLE 2. Effect of calcium ion on sporulation and intracellular proteinase activity in wild-type strains and a mutant of *B. subtilis* lacking ISP-1

Strain	Ca^{2+} concn in medium	No. of spores at t_{15} (turbidity[a])	Intracellular azocaseinase (ΔA_{335}/h per mg)[b] at t_{10}
168 (*trpC2*)	<2 μM	1.9 (±0.8) × 10^6	—[c]
	1.4 mM	1.5 (±0.7) × 10^8	—
168I	<2 μM	N.D.[d]	0.30
	1.4 mM	3.2 (±0.8) × 10^8 (254)	0.38
3036 (ISP-1⁻)	<2 ΓM	N.D.	0.036
	1.4 mM	4.9 (±0.4) × 10^8 (303)	0.096

[a] Turbidity is reported as Klett units with a no. 66 filter.
[b] Average values for duplicate assays from duplicate cultures.
[c] —, Not determined.
[d] N.D., Not determined quantitatively; however, when examined with a phase-contrast microscope at $t_{10.5}$, all cultures containing 1.4 mM Ca^{2+} had refractile bodies, whereas all cultures with <2 μM Ca^{2+} had no detectable refractile bodies.

To determine whether the inhibition of protein degradation by calcium ion limitation might be due to an effect of these ions on proteinase activity directly, we assayed levels of total proteinase activity in soluble cell extracts of cells grown in the presence and absence of calcium ions (Fig. 4). Evidently, calcium ions must be present in the growth medium in order to produce normal synthesis and/or activation of ISP-1, which accounts for about 80% of the total proteinase activity in the soluble fraction (5, 29). The data in Table 2 confirm that in Ca^{2+}-containing CDSM medium, ISP-1 accounted for about 74% of the total azocaseinase activity in the cells at t_{10}. In contrast, in the same cultures, the production of total extracellular proteinase (azocaseinase) activity was reduced by only 20% in cultures lacking added Ca^{2+} compared with the control cultures (data not shown).

Nakamura et al. (29) have described an asporogenous mutant of *B. subtilis* which lacks the ability to degrade intracellular protein. The mutant was also found to be deficient in calcium ion uptake. The cells were able to degrade protein at the wild-type rate when suspended in a buffer solution containing both calcium ions and 1% toluene. We reasoned that if the energy inhibitors acted strictly by blocking calcium uptake, which is energy dependent in vegetative cells (7), toluene might reverse the effects of the energy poisons on the rates of proteolysis (8). In the presence of 10 μM CCCP, the addition of 0.1% toluene had no effect on the inhibition of the rates of proteolysis caused by the CCCP, but the addition of 1% toluene increased rates of degradation to well above those of even the uninhibited control cultures. Since exposure of cultures to 1% toluene for only 2.5 h caused the turbidities to increase from 350 (in control cultures) to 588 Klett units and the medium to become yellow and somewhat viscous, we do not believe that the apparent effect on proteolysis can be meaningfully interpreted.

As an alternative to the toluene treatments, we attempted to overcome the energy block by adding the calcium ionophore calimycin (A23187) to CCCP-inhibited cultures to allow passive diffusion of the calcium ions (1.4 mM in the medium) into cells. At 1.0 μM, calcimycin had no effect on either the turbidities of the cultures or the rates of proteolysis.

Finally, we examined the effects of two of the energy inhibitors, arsenate and CCCP, and the protein synthesis

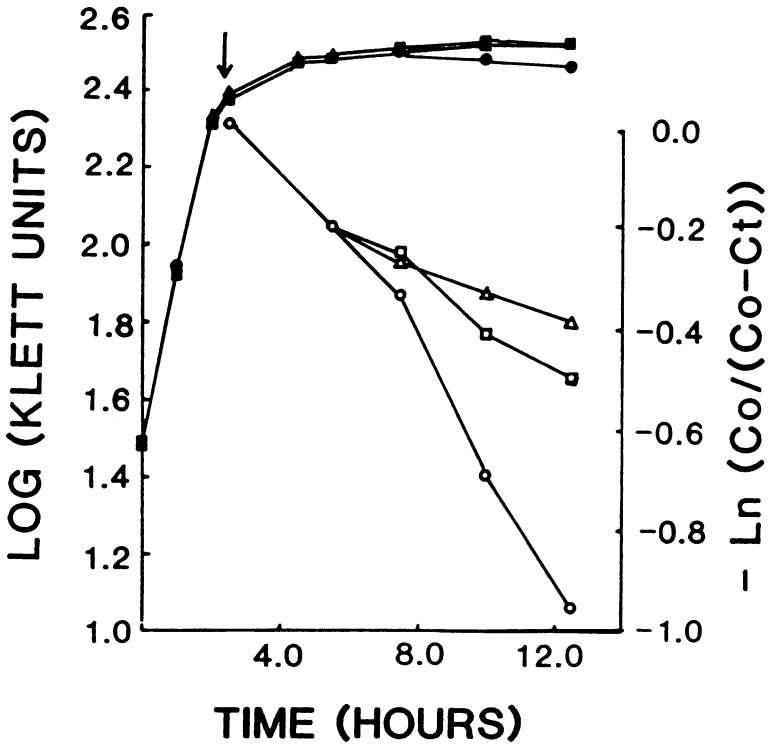

FIG. 3. Comparison of growth and proteolysis of *B. subtilis* cells grown in normal CDSM, Ca^{2+}-deficient CDSM, or Ca^{2+}-deficient CDSM containing 200 μM dibromo-BAPTA. Cells were grown and labeled as described in the legend to Fig. 1. The cells were suspended in normal or Ca^{2+}-deficient CDSM and allowed to sporulate. Duplicate 1.0-ml samples were removed from duplicate flasks at t_0, t_3, t_5, $t_{7.5}$, and t_{10}. Dibromo-BAPTA (200 μM) was added at t_2 (arrow). Symbols: ●, growth of control cells (normal CDSM); ■, growth of cells in Ca^{2+}-deficient CDSM; ▲, growth of cells in Ca^{2+}-deficient CDSM to which dibromo-BAPTA was added at t_2; ○, degradation of protein in control cells (normal CDSM); □, degradation of protein in cells in Ca^{2+}-deficient CDSM; △, degradation of protein in cells in Ca^{2+}-deficient CDSM to which dibromo-BAPTA was added at t_2.

inhibitor chloramphenicol at levels which strongly inhibited proteolysis in vivo on the ability of cells to take up calcium ions under the same conditions as those used to study proteolysis (Fig. 5). Clearly, none of the inhibitors had appreciable effects on the rate or extent to which cells accumulated Ca^{2+}. Chloramphenicol (added at t_3) had a slight effect on the extent of calcium ion uptake.

Proteolysis in a mutant lacking ISP-1. From the study of the mutant of *B. subtilis* which was defective in Ca^{2+} uptake, Nakamura et al. (29) suggested that the failure to degrade protein might be due to the inability of ISP-1 to be fully expressed or fully active. This suggestion is indeed consistent with the results shown in Fig. 4. To test this idea more rigorously, we have studied the effects of calcium ions on rates of degradation of protein in a mutant of *B. subtilis* containing an insertionally inactived gene for ISP-1, isolated by Band et al. (1).

As they reported previously for cells grown in nutrient broth, we found (Table 2) that the mutant cells grown in CDSM contained less total proteinase (1) than the parent cells but sporulated to the same extent (1, 21). The apparent differences caused in proteinase levels by the presence and absence of Ca^{2+} in the parental strain 168I (Table 2) compared with the differences caused in our standard strain (Fig. 4) are due primarily to the earlier harvest time (t_{10}) used for 168I. The mutant cells also underwent less lysis during sporulation (1) (Table 2) than the parent cells; indeed, this was the only obvious physiological response to the absence of the ISP-1. We found that the mutant cells degraded intracellular proteins at rates which were indistinguishable from those of the parental strain until after t_8 (Fig. 6), whereas Band et al. (1) have reported that the mutant appears to degrade protein in nutrient broth medium at a rate consistently slower than that of the parental strain. However, just as we found for our standard strain (see above), we found that a deficiency of calcium ions in the medium (levels of <2 μM) inhibited the degradation of protein in both the mutant and parental strains (data not shown). These results strongly suggest that calcium ion is needed for some process in proteolysis other than ensuring the activity of ISP-1.

DISCUSSION

For more than 30 years, intracellular protein degradation in mammalian cells as well as bacterial cells has been suggested to be energy dependent, primarily because metabolic poisons of energy production were found to inhibit proteolysis (12). The likelihood that this energy dependency was direct rather than indirect has increased with the discovery and characterization of specific elements of proteolysis which require ATP hydrolysis, such as the ubiquitin conjugation system and various ATP-dependent proteinases (3, 6, 27, 37).

In bacteria, a number of ATP- or energy-dependent proteolytic events have been found. In *E. coli* cells, several distinct processes of proteolysis clearly occur (27, 32, 47), and their energy requirements differ. The assignment of specific proteinases to specific types of proteolysis is far from accomplished. For example, studies on the well-characterized ATP-dependent proteinase La have shown that

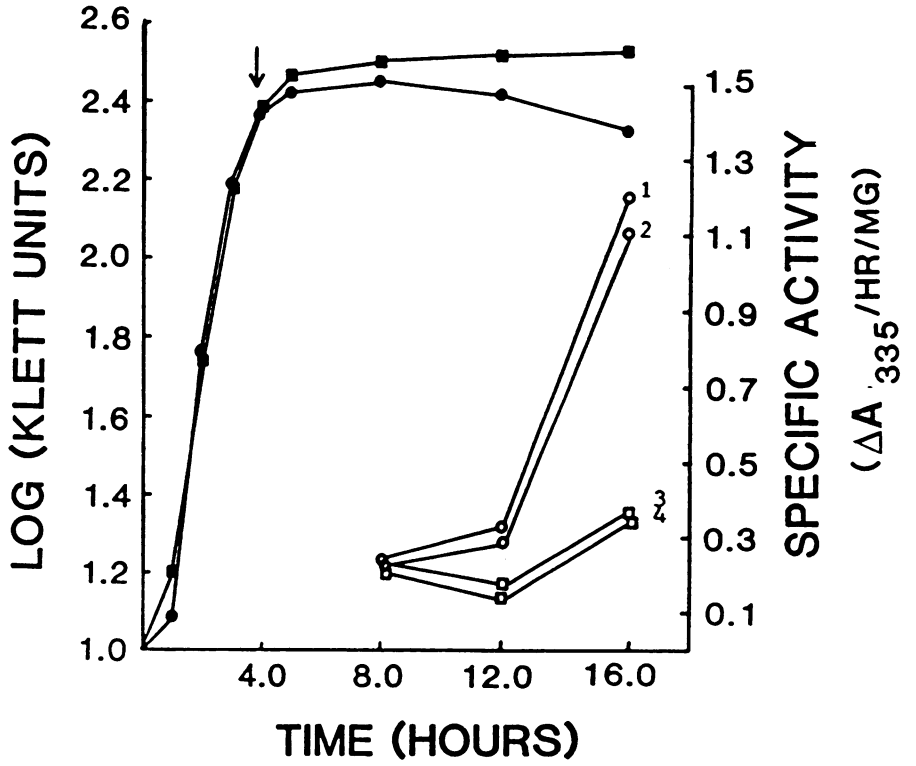

FIG. 4. Comparison of the total specific activity of intracellular proteases from *B. subtilis* cells grown in normal or Ca^{2+}-deficient CDSM. Several colonies of *B. subtilis* 168 growing on a gelatin plate were used to inoculate 500 ml of CDSM in a 2.0-liter flask. The culture was incubated at 37°C in a New Brunswick G-25 rotary shaker-incubator at 220 rpm until the turbidity of the culture reached 50 to 200 Klett units (10 to 12 h). A 10% inoculum was transferred into 2.0-liter flasks containing 500 ml of fresh CDSM, half of which were deficient in Ca^{2+}. The cells were allowed to sporulate; at t_4, t_8, and t_{12}, duplicate sets of flasks in which cells grown in the presence or absence of Ca^{2+} were harvested and centrifuged for 10 min at 4°C and 8,000 rpm (10,440 × *g*). The culture broth was decanted, and the cells were washed first with 50 ml of cold 0.05 M sodium acetate–0.1 M KCl, pH 6, and then with 50 ml of cold 0.05 M $NaHCO_3$–1 M KCl, pH 10, to remove extracellular proteinases. The washed pellets were stored in a freezer at −20°C. The samples were thawed the following day at room temperature. Each sample was suspended in 0.05 M $NaHCO_3$, pH 10, to a volume of three times the pellet volume. The cell suspension was passed twice through a 50-ml French pressure cell at 20,000 lb/in^2 and 0°C. The cells were then centrifuged in a Sorvall RC-2B refrigerated centrifuge with an SS-34 rotor at 15,000 rpm and 4°C for 1 h. Each supernatant fraction was then divided and placed in dialysis tubing; one half of the sample was placed into 0.05 KH_2PO_4–1 mM $CaCl_2 \cdot 2H_2O$, pH 6, and the other half was placed in the same buffer but without the calcium salt. The extracts from the cells grown in the presence of Ca^{2+} were buffered separately from the cells grown in the absence of Ca^{2+}; all samples were dialyzed overnight with two changes of buffer, and the following morning they were assayed for azocasein hydrolyzing activity. Symbols: ●, growth of cells in normal CDSM; ■, growth of cells in Ca^{2+}-deficient CDSM; ○, specific activity of cells grown in normal CDSM (1, no Ca^{2+} in dialysis buffer; 2, Ca^{2+} in dialysis buffer); □, specific activity of cells grown in Ca^{2+}-deficient medium 3, no Ca^{2+} in dialysis buffer; 4, Ca^{2+} in dialysis buffer).

this enzyme can account for no more than half of the degradation of abnormal protein and accounts for none of the degradation of bulk proteins during cell starvation (26, 27). Another ATP-dependent proteinase, which has multiple subunits, has been isolated and characterized by Maurizi and his colleagues (20); this very interesting enzyme appears to be a major intracellular proteinase in *E. coli* cells and has been called Ti protease by Goldberg and co-workers (15, 16).

The degradation of protein in cells of *Bacillus* species undergoing sporulation is four to five times more rapid (2) and more extensive (40) than that which occurs in other types of bacteria which are subjected to starvation (35). Presumably, protein degradation during sporulation has the biological function of allowing spore development and not primarily of avoiding death of the mother cell. At the onset of sporulation, several enzymes of *B. subtilis* are preferentially degraded in an energy-dependent manner (24, 38, 49), but no studies have been done previously on the possible energy dependence of bulk protein degradation during sporulation. All of the energy poisons used here, which generally had slight or no effect on cell lysis, inhibited intracellular protein degradation. Although the mechanism of the energy requirement is not certain, the results with arsenate suggest that ATP itself may be required for proteolysis. This conclusion is consistent with the fact that DCCD at the level used here (100 μM) completely depletes ATP pools in vegetative cells but reduces the proton motive force by only half (19). The most potent inhibitor, CCCP, was effective here at concentrations much lower than those used to induce lysis of vegetative cells (19). While CCCP of 5 μM was reported to drastically inhibit calcium ion uptake in membrane vesicles prepared from vegetative cells (7), at 100 μM it was found to actually stimulate calcium ion uptake in sporulating *B. subtilis* cells (8). In any case, when we studied the effects of CCCP on calcium uptake under the same conditions used to study proteolysis, we found that it did not slow uptake. This result, taken with the observed requirement for calcium ion for sporulation and for maximal proteolysis in the ISP-1 mutant, led us to the conclusion that both energy (possibly ATP) and Ca^{2+} are required for maximal

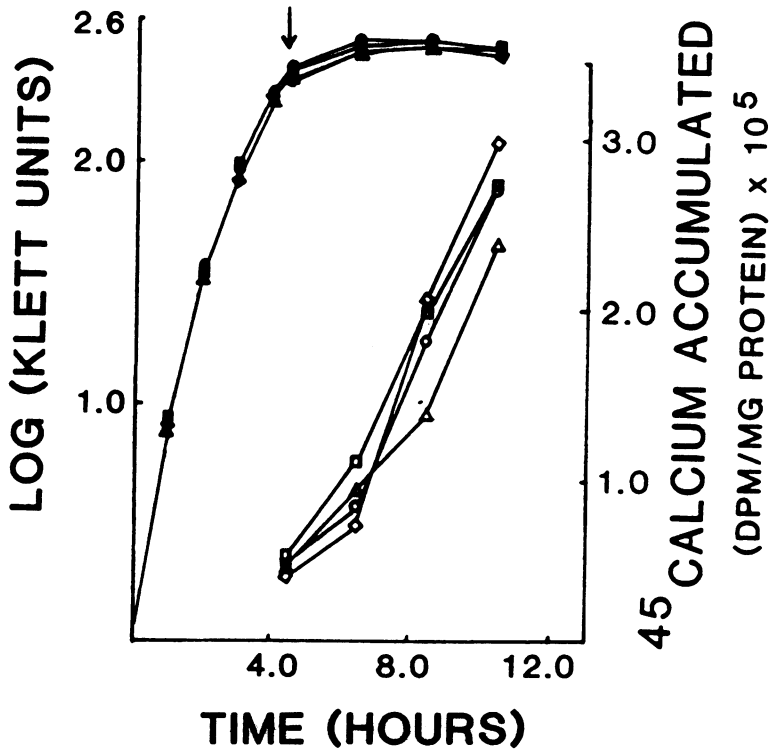

FIG. 5. Effect of arsenate, chloramphenicol, and CCCP on Ca^{2+} uptake and accumulation of *B. subtilis* cells. An inoculum of *B. subtilis* cells was prepared as described in the legend to Fig. 4. A 10% inoculum was transferred into 300-ml flasks containing 75 ml of fresh CDSM supplemented with 0.7 mM $CaCl_2 \cdot 2H_2O$. After 1 h, $^{45}CaCl_2 \cdot 2H_2O$ was added to each flask to a final concentration 0.4 μCi/ml. The cells were allowed to sporulate, and at t_3, 4 mM arsenate, 100 μg of chloramphenicol per ml, or 10 μM CCCP was added. At t_0 (indicated by the arrow), t_2, t_4, and t_6, duplicate 1.0-ml samples of cells were removed from each duplicate flask and washed with warm CDSM containing an excess (5.0 mM) of unlabeled calcium chloride. The cells were treated with 1.25 ml of 10% TCA, and the samples were incubated at 37°C in a New Brunswick rotary shaker-incubater at 220 rpm for 2 h. Samples were spun at 12,000 rpm (9,000 × *g*) for 5 min, and the supernatant fraction was saved; the radioactivity in 0.3 ml of this fraction was counted in duplicate. Symbols: ●, growth of control cells; ◆, growth of cells to which arsenate was added; ▲, growth of cells to which chloramphenicol was added; ■, growth of cells to which CCCP was added; ○, amount of $^{45}Ca^{2+}$ taken up by control cells; ◇, amount of $^{45}Ca^{2+}$ taken up by cells to which arsenate was added; △, amount of $^{45}Ca^{2+}$ taken up by cells to which chloramphenicol was added; □, amount of $^{45}Ca^{2+}$ taken up by cells to which CCCP was added. Note: 10^5 dpm/mg of protein ≅ 5.4×10^{-2} mmol of Ca^{2+} per 10^{12} cells.

degradation of intracellular proteins during sporulation. It must be stressed that either or both of these apparent requirements may be indirect effects which have their impacts on the proteolytic system through secondary agents.

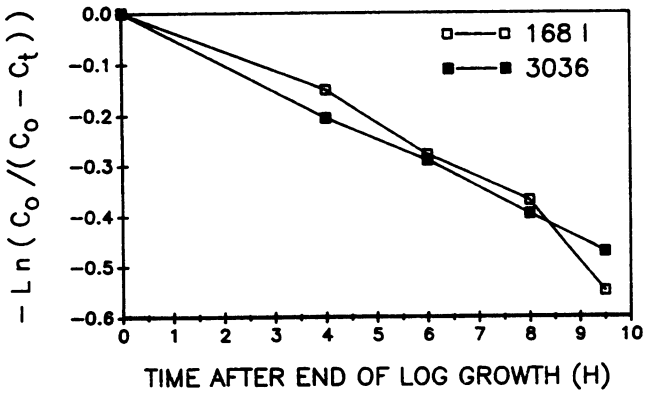

FIG. 6. Degradation of intracellular protein in a strain deficient in ISP-1 (*B. subtilis* 3036) compared with the parental strain (*B. subtilis* 168I). Experiments were conducted as described in legend to Fig. 1.

Earlier attempts to demonstrate stimulation of proteolysis by ATP in crude extracts of sporulating cells revealed slight or no stimulation (Geele and Hageman, unpublished observations).

With regard to the Ca^{2+} requirement, calmodulinlike proteins (10, 18, 34, 42) and other calcium-binding proteins (17, 48) have been found in several genera of procaryotic cells. Whether the calmodulinlike protein in *B. subtilis* (10) is important for proteolysis is not known, but preliminary experiments in our lab have shown that the calmodulin antagonist trifluoperazine selectively blocks sporulation of *B. subtilis* cells (M. T. Ramos and J. H. Hageman, unpublished observations). The requirement of cells for Ca^{2+} to achieve maximal rates of proteolysis may be an indirect effect, but proteolysis is the earliest event in sporulation so far reported on which Ca^{2+} has an impact.

The inhibition of proteolysis by chloramphenicol even when added rather late into sporulation is somewhat surprising; one might interpret this as the continuous need for the synthesis of proteinase. On the other hand, growth of *E. coli* or *Salmonella typhimurium* cells in the presence of concentrations of chloramphenicol 100 times lower than those used here makes several enzymes much more sensitive to derepression and also increases nucleotide pools, apparently by

increasing RNA turnover (9). These results point out that the drug may have actions other than the inhibition of protein synthesis. As a further alternative, we tentatively suggest that chloramphenicol may itself be a proteinase inhibitor.

Finally, we confirm that the major intracellular proteinase in the soluble fraction of *B. subtilis* cells is dispensable for sporulation and has no detectable effect in the measured rate of proteolysis until after t_8 (Fig. 6). We suggest that the slight difference in rates of proteolysis between the parental strain and the mutant with the insertionally inactivated ISP-1 reported by Band et al. (1) was due to the lysis observed in the parental strain (see Fig. 2 of reference 1), for which no correction was apparently made. Although the correction for lysis method we have applied is no doubt flawed, we believe it is a conservative one. It may be desirable to develop some new methods for measuring protein degradation, especially when cell lysis occurs.

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