JOURNAL OF BACTERIOLOGY, Jan. 1986, p. 139–145
0021-9193/86/010139-07$02.00/0

Vol. 165, No. 1

Activation of Intracellular Serine Proteinase in *Bacillus subtilis* Cells during Sporulation

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Received 15 July 1985/Accepted 1 October 1985

Cells of *Bacillus subtilis* 168 (*trpC2*) growing and sporulating in a single chemically defined medium carried out intracellular protein degradation and increased their levels of intracellular serine protease-1 in a manner very similar to what had previously been reported for cells sporulating in nutrient broth. The results were interpreted to mean that these processes are intrinsic to sporulation rather than medium dependent. To determine the cause of these increases in specific activity of proteinases, we purified the protease, prepared rabbit immunoglobulins directed against it, and monitored changes in protease antigen levels by performing rocket immunoelectrophoresis. In cells sporulating in nutrient broth, the protease antigen levels increased about 7-fold, whereas the specific activity increased about 150-fold, for an activation of about 20-fold. In cells sporulating in the single chemically defined sporulation medium, the protease antigen increased about 10-fold, whereas the specific activity increased at least 400-fold, for an activation of about 40-fold. These results were interpreted to mean that a posttranslational event activated the protease in vivo; a previously described endogenous proteinase inhibitor was confirmed to be present in the strain used. Chloramphenicol added to the cultures inhibited both the increases in antigen levels and in the specific activity of the proteinase.

Bacillus subtilis cells produce at least two intracellular serine proteinase activities which are referred to as ISP-1 (15, 31, 38) and ISP-2 (37). Both ISP-1 and ISP-2 (7, 31, 37) increase in specific activity during sporulation, but ISP-1 appears to account for at least 80% of the intracellular azocasein or azocollagen hydrolyzing activity (11, 36; S. A. Smith and J. H. Hageman, unpublished data). These two proteinases are distinct from the three proteinases excreted by *B. subtilis* cells during sporulation (18, 30) and from other cytoplasmic or membrane-associated proteinases (20, 34).

Possible roles of proteinase activity in protein degradation in *B. subtilis* cells have been considered for some time (19, 36), and evidence for such roles was reviewed most recently by Maurizi and Switzer (23). Genetic and physiological evidence has been presented for a role of ISP-1 in bulk protein degradation during sporulation (15, 27), and evidence has been reported that mutations affecting ISP-2 (12, 32) also result in impaired ability of cells to degrade intracellular proteins during sporulation. In most previous studies (21, 29), workers have attempted to correlate intracellular proteinase activity and protein degradation in cells sporulating in complex or semidefined media. We show in this study that cells sporulating in a chemically defined sporulation medium (CDSM) (9) also elaborate intracellular proteinase and degrade intracellular proteins efficiently.

Two to three hours after the onset of sporulation of *B. subtilis* cells in nutrient broth, ISP-1 undergoes a rapid increase in specific activity (7, 29, 31); such an increase might be due to either synthesis of a new enzyme or activation of a preexisting enzyme. Results of qualitative immunological study by Shaginyan et al. (33) have suggested that the increase in specific activity is accounted for by de novo synthesis. On the other hand, the earlier finding of Millet (25) and Millet and Gregoire (26) that ISP-1 is specifically inhibited by an intracellular protein inhibitor (M_r = 15,500) that disappears from the cells at the same time that the ISP-1 activity increases suggested that activation of ISP-1 activity, in excess of newly synthesized protein, might be expected. We examined the question of de novo synthesis of ISP-1 in cells sporulating in both nutrient broth and CDSM using quantitative rocket immunoelectrophoretic methods. While some increase in ISP-1 antigen does occur, the majority of the increase in specific activity of this proteinase is due to some form of enzyme activation.

MATERIALS AND METHODS

Chemicals. All bacterial culturing media and Freund adjuvants were obtained from Difco Laboratories (Detroit, Mich.). All inorganic salts used in growth media were from Mallinckrodt, Inc. (St. Louis, Mo.) and were reagent grade. DEAE-cellulose was from Whatman, Inc. (Clifton, N.J.).

Reagents used in electrophoreses were acrylamide and *N,N'*-methylene-bisacrylamide (electrophoresis grade) from Aldrich Chemical Co., Inc. (Milwaukee, Wis.); ammonium persulfate from J. T. Baker Chemical Co. (Phillipsburg, N.J.); sodium dodecyl sulfate, *N,N,N',N'*-tetramethylethylenediamine from Sigma Chemical Co. (St. Louis, Mo.); and Coomassie brilliant blue R-250 and silver staining kit from Bio-Rad Laboratories (Richmond, Calif.). Protein Assay Kit I and agarose instant gel tablets were from Bio-Rad Laboratories. All other enzymes and biochemical were the highest grade available and were obtained from Sigma.

Bacterial strains: growth and harvesting. Studies were conducted with *B. subtilis* 168 (*trpC2*) and *B. subtilis* 168 S-87, a pleitropic mutant deficient in several proteinases, including the major intracellular enzyme (8). Stock cultures of each were maintained as described previously (7). Nutrient broth medium was that described by Hanson et al. (10). For all experiments, 70 ml of growth medium in a 300-ml baffled flask (Bellco Glass, Inc., Vineland, N.J.) was inoculated with a colony from a stock plate and incubated at 30°C with shaking (250 rpm) for 12 to 13 h in a New Brunswick S-86 water bath shaker. A 5% inoculum was then transferred to fresh medium (no more than 500 ml in a 2-liter baffled

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flask; Bellco) and incubated at 37°C with shaking (250 rpm) in a New Brunswick G-25 rotary shaker-incubator. After 2.5 h, a 5% inoculum was transferred into 475 ml of fresh medium in 2-liter baffled flasks, and growth was continued under the same conditions.

The growth of the cultures was monitored turbidometrically with a Klett-Summerson colorimeter with a no. 66 filter. Cells were routinely harvested 4 to 6 h after the end of logarithmic growth (t_4 to t_6, respectively), with a CEPA Laboratory Centrifuge, type LE, or by centrifugation, bringing samples to 8,000 rpm (10,400 × *g*) in a Sorvall RC-2B refrigerated centrifuge in a GSA rotor. The cell paste was immediately frozen with a dry ice-acetone bath and stored at −20°C.

Growth of cells in CDSM was carried out as described previously (9).

Protein degradation measurements. Degradation of protein in cells grown in CDSM was measured by a method similar to that of Spudich and Kornberg (36). Experiments were started by transferring a 2% inoculum to 50 ml of fresh CDSM, containing 20 μg of L-leucine and 50 μg of L-valine per ml; growth was continued at 37°C in 300-ml triple-baffled sidearm flasks (Bellco Glass). After 2.5 h of growth, 25 μCi of 4,5-[^{3}H]L-leucine (55 Ci/mmol; ICN Pharmaceuticals Inc., Irvine, Calif.) was added and growth was continued. At the end of the log phase of growth (t_0, ~4.5 h after inoculation), 2.5 mg of unlabeled L-leucine per ml was added as a chase. Cells (1 ml) were removed from the culture periodically, treated with 0.25 ml of ice-cold, 50% (wt/vol) trichloroacetic acid, and centrifuged at 9,000 × *g* for 10 min in a Beckman microfuge. The pellet was washed with 0.75 ml of 50% (wt/vol) trichloroacetic acid, the supernatant solution was decanted, and the pellet was suspended in 1.0 ml of 0.05 M NaOH by repeated mixing in a vortex shaker over a 30-min period at 22°C. Samples of 0.05 or 0.1 ml were removed, added to 10 ml of 3a70B counting solution (RPI Corp.), placed in the dark for 2 h, and counted in an LS100C liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

Proteinase assays. Intracellular serine protease activity was routinely measured by a method similar to that of Geele et al. (7). A 50- to 200-μl portion of enzyme solution was incubated at 37°C in the presence of 1% azocasein, 2 mM $CaCl_2$, and 67 mM Tris hydrochloride (pH 7.8) in a total volume of 1.5 ml for 4 h. Reactions were stopped by adding of 2.0 ml of 10% (wt/vol) trichloroacetic acid; precipitates were removed by centrifugation for 15 min at 2,600 rpm (1,100 × *g*) in a Sorvall GLC-2 benchtop centrifuge, and A_{335} of the supernatant fraction was measured in a Beckman model 24 spectrophotometer. All assays were done in duplicate or triplicate. Inhibitors were preincubated with the enzyme solution for at least 15 min at room temperature before they were assayed.

Azocollagenase activity was measured by a method similar to that described in the technical bulletin "Azocoll," available from Calbiochem-Behring, La Jolla, Calif. Azocoll (0.25 g) was suspended in 50 ml of 0.05 M Tris hydrochloride–1 mM $CaCl_2$ (pH 7.8) (Tris-Ca buffer) by stirring rapidly with a magnetic stirrer. Fractions of this suspension (0.5 ml each) were transferred to 1.5-ml polyethylene microfuge tubes with a 1.0-ml disposable syringe without a needle. Enzyme solutions (20 μl) was added to the suspension, and the tubes were incubated at 37°C in a water bath. After 1.5 to 2 h, the reaction was stopped in an ice water bath, and the solid substrate was removed by centrifugation at 9,000 × *g* for 2 min in a microfuge. The A_{520} was measured in the supernatant solutions. Incubation times of less than 1.5 to 2 h were not linear with time when low enzyme concentrations were used; thus, assay conditions had to be developed so that all samples assayed were linear with both enzyme concentration and time. Subsequently, we developed an improved azocollagenase assay (3).

Protein assays. Protein was estimated by the dye-binding assay of Bradford (2) with the Bio-Rad Protein Assay Kit I with an immunoglobulin standard. In some cases, when the protein concentrations were 0.01 to 0.1 mg/ml, the protein concentration was determined by the method of Kalb and Bernlohr (13).

Isolation of ISP-1. The isolation of ISP-1 was carried out by a method similar to that described by Strongin et al. (38). *B. subtilis* 168 cells harvested at t_5 (approximately 100 g [wet weight]) were washed once with 4 to 5 volumes of iced 0.05 M sodium bicarbonate (pH 10.0), containing 1 M KCl, and once with iced Tris-Ca buffer. After the final wash, the cells were suspended to a total volume of 280 ml in Tris-Ca buffer. All further purification steps were carried out at 4°C unless otherwise stated.

Cells were broken by two passes through a chilled French pressure cell (50 ml) at 25,000 lb/in^2. Cell debris was removed by centrifugation at 30,000 × *g* for 1 h. The crude soluble extract was decanted and treated with 0.12 volumes of a solution containing 1 mg of DNase I per ml, 0.5 mg of RNase A per ml, 50 mM $MgCl_2$, and 0.5 M Tris (pH 7.0); this solution was stirred for 2 to 4 h at 4°C. The extract was dialyzed against Tris-Ca buffer overnight and fractionated with ammonium sulfate. Protein precipitating between 40 and 85% saturation with $(NH_4)_2SO_4$ at 0°C (5) was suspended in the Tris-Ca buffer and dialyzed against the same buffer. Chromatography on DEAE-cellulose and on gramicidin S-Sepharose was by the method of Strongin et al. (39), except that the gramicidin S-Sepharose column was washed extensively with 50% dimethylformamide before use to remove unbound gramicidin S-Sepharose. Active proteinase fractions from the gramicidin S-Sepharose column were further purified by electrophoresis in tube gels (T. Burnett, Ph.D. dissertation, New Mexico State University, Las Cruces, 1984) followed by electroelution by the technique of Braatz and McIntire (1) into Tris-Ca buffer containing 30% (vol/vol) glycerol and stored at −20°C.

The protein pooled fraction from electroelution was examined by electrophoresis on 10% polyacrylamide gels. A major protein band was found at an R_f of 0.51, and a very minor protein band was found at an R_f of 0.54. Electrophoresis of the major band in the presence of 0.1% sodium dodecyl sulfate revealed major protein bands at M_r 28,000 and 29,500, with a minor component at 56,000. Upon sodium dodecyl sulfate-gel electrophoresis, the trace component of the R_f of 0.54 revealed proteins of the same molecular weight, but with the 56,000 M_r form being the major component. These multiple electrophoretic forms are consistent with the findings reported by others (28, 39).

Preparation of antibodies. Standard methods (41) were used to prepare antiserum from 4-month-old New Zealand white rabbits after injection of a total of 65 μg of ISP-1 purified by preparative electrophoresis.

Immunoglobulins were separated from the serum by $(NH_4)_2SO_4$ fractionation and DEAE-cellulose chromatography, as described by Garvey et al. (6); immunoglobulin fractions were concentrated in an Amicon model 202 ultrafiltration cell with a YM-10 filter and stored frozen at −20°C until use.

Immunoelectrophoresis. For qualitative immunoelectro-

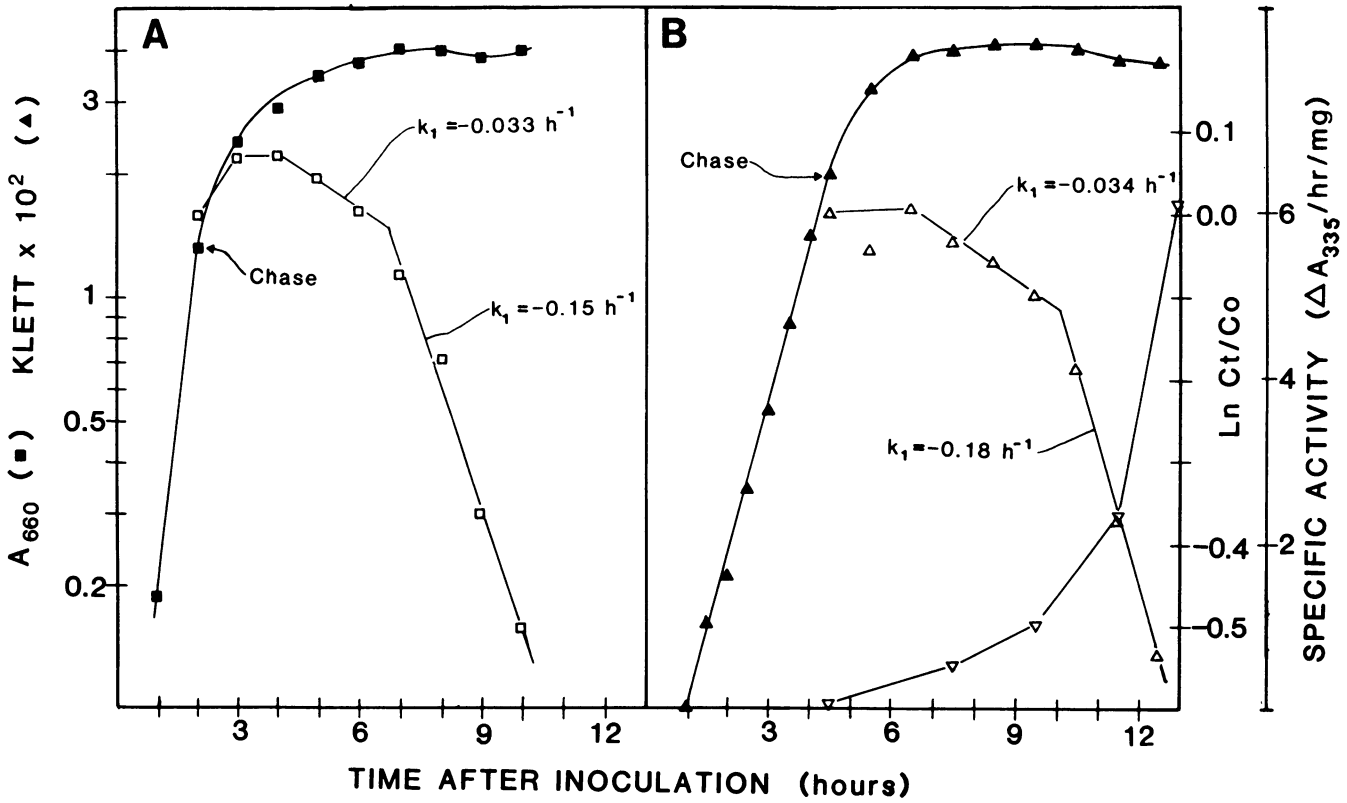

FIG. 1. Degradation of intracellular protein by *B. subtilis* growing in nutrient broth or CDSM. Cells were labeled during log phase growth with [^{3}H]phenylalanine (A) or [^{3}H]leucine (B) and chased at t_0 (arrows) with an excess of unlabeled phenylalamine or leucine, respectively. Acid-precipitable counts per milliliter of culture during the sporulation period were determined as described in the text and are expressed as the Naperian log function of the ratio of counts at time t (C_t) to counts at time t_0 (C_0) so that the slope of this line as a function of time represents an apparent first-order rate constant. Data shown in panel A are values taken from Fig. 2 of reference 36 for cells grown in nutrient broth, which were replotted in Naperian form. Data in panel B are for cells grown in CDSM and also show levels of intracellular azocaseinase activity (▽) in parallel cultures of cells; values are averages of duplicate cultures.

phoresis, proteins of *B. subtilis* 168 were electrophoresed on discontinuous 10% polyacrylamide gels as described above, and in detail elsewhere (Burnett, Ph.D. dissertation).

Rocket immunoelectrophoresis was performed by the procedure of Weeke (44) with some modifications described by Mayer and Walker (24). The buffered agarose gel used in these experiments was prepared with Bio-Rad agarose instant gel tablets: for every 5.0 ml of agarose needed, one tablet was suspended in deionized water (5.0 ml) in a test tube (18 by 175 mm), boiled for 5 min, and cooled to 50 to 55°C in a water bath until use. A uniform gel (1.5-mm thick) was poured onto a glass plate (100 by 100 mm) as described by Weeke (44). For some experiments, a contact gel was poured on one end of the plate. For studies with crude cell extracts, elongated wells were found to provide the best results.

After electrophoresis of the antigen into the antibody-containing agarose slab, the gel was removed, blotted thoroughly with filter paper, and soaked in two washes of normal saline (200 ml, 1 h each) and then deionized water (400 ml, 1 h). The gel was again blotted and dried to a thin film at 65°C. Immunoprecipitate lines were stained by placing the dried gel in 0.05% Coomassie brilliant blue R-250 in 50% methanol–5% acetic acid for 10 to 30 min. The gel was destained in 10% ethanol–7% acetic acid and dried at 65°C.

Rocket heights were measured from the top of each well to the top of the precipitin lines. Rocket height was found to be proportional to antigen over a 10-fold concentration range (from 5 to 25 mm). To establish the linearity of rocket height to antigen concentration, a series of ISP-1 dilutions were run in triplicate and plotted as a function of rocket height; the plot of the average values was highly linear with a correlation coefficient of 0.99.

Crossed immunoelectrophoresis. Crossed immunoelectrophoresis was performed as described by Weeke (44) or with modifications as described by Mayer and Walker (24). Antigens were separated in the first dimension by electrophoresis on 10% disk-polyacrylamide gel electrophoresis microslab gels or on agarose gels (9.0 V/cm for 1.5 h) and then into the antibody-containing gel. A Plexiglas (Rohm & Haas Co., Philadelphia, Pa.) electrophoresis chamber was designed and built so that six crossed immunoelectrophoresis plates could be run simultaneously to keep electrophoretic conditions as uniform as possible. A low voltage (2.0 V/cm) was applied as described above for 6 to 14 h. After electrophoresis, the plate was pressed, washed, dried, and stained as described above.

Tandem-crossed immunoelectrophoresis. Tandem-crossed immunoelectrophoresis was performed by the method of Kroll (16) with modifications as described by Mayer and Walker (24).

RESULTS

Protein degradation and proteinase formation by *B. subtilis* cells grown in CDSM. Recently, we developed CDSM which

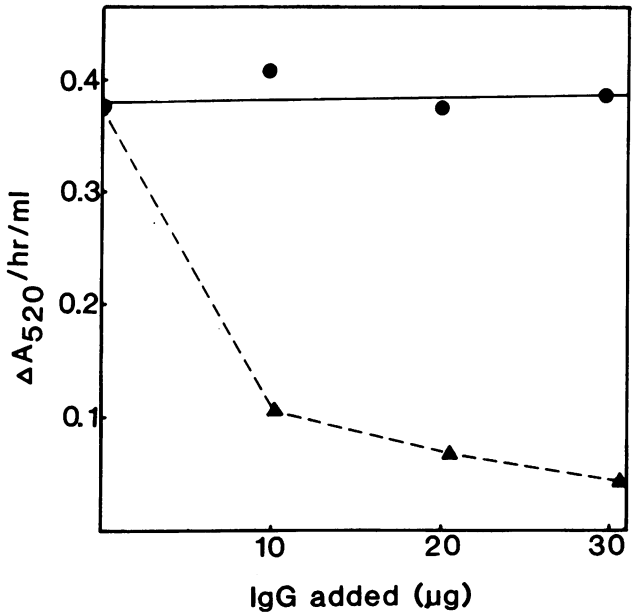

FIG. 2. Inhibition of azocollagenase activity of purified ISP-1 by monospecific immunoglobulins. IgG fractions were obtained from preimmune rabbit (●) and from serum following injections with ISP-1 (▲) (see the text) and used to titrate ISP-1 recovered from a second passage through a gramicidin S-Sepharose affinity column. ISP-1 (2.4 μg) was preincubated with the indicated amounts of immunoglobulins for 1.5 h at 37°C and an additional 1.0 h at 4°C in a total volume of 0.1 ml of Tris-Tricine immunoelectrophoresis buffer (80 mM Tris, 24 mM Tricine, 0.3 mM calcium lactate, 0.04% [wt/vol] sodium azide, adjusted to pH 8.6 with HCl) containing 1 mM $CaCl_2$. Preincubation solutions were centrifuged, and 40 μl of each supernatant solution was assayed in duplicate for azocollagenase activity (see the text).

allows *B. subtilis* cells to grow rapidly and undergo a high degree of sporulation (9). Cells grown in CDSM were found to degrade intracellular protein in a manner very similar to those grown in nutrient broth (Fig. 1). Cells growing in CDSM also began producing azocasein hydrolyzing activity at about the same time as those growing in nutrient broth (7), but they produced somewhat larger amounts when grown in CDSM than in nutrient broth (data not shown). It appears that the complex of amino acids, peptides, and proteins present in nutrient broth have little effect on the elevated rate of intracellular protein catabolism but may repress slightly the level of intracellular protease(s) in *B. subtilis* cells undergoing sporulation. Thus, the elevation in the protease activity appears to be an intrinsic event in sporulation (or stationary-phase growth) and is not simply induced by exogenous substrates.

These observations raise an important fundamental question: What mechanism accounts for the increases in proteinase activity during sporulation? We attempted to answer that question using quantitative immunological techniques.

Preparation and characterization of antibodies against intracellular proteinase. Reysset and Millet (31) first described and partially purified an ISP (subsequently termed ISP-1) present in *B. subtilis* cells; later ISP-1 was shown to account for nearly all the casein (8) and azocoll (29, 37) hydrolyzing activity in sporulating cells of *B. subtilis*. In 1979, two groups (15, 38) described methods of purifying ISP-1 to homogeneity and confirmed its identity as a Ca^{2+}-stabilized-requiring metalloserine proteinase (40). We purified ISP-1 by a modification of the procedure of Strongin et al. (38) as described above. The protein was used to prepare an immunoglobulin G (IgG) fraction of antibodies from rabbits as described above.

Because of the minor protein contaminant in the proteinase preparation, two experiments were done to establish the uniqueness of the anti-ISP-1 IgG antibody fraction. First, titration of a highly purified fraction of ISP-1 with the antibodies raised against it caused a maximum of 90% inhibition of the protease activity (Fig. 2). Additionally, a highly purified fraction of ISP-1 (two passes through a gramicidin S-Sepharose column) was prepared and used as a standard in a crossed immunoelectrophoresis analysis. The protease was first electrophoresed on a microslab gel of 10% polyacrylamide in three lanes, with each containing 1 μg of ISP-1. One track was cut out and embedded in a plate of agarose containing anti-ISP-1 IgG antibodies (see above) and electrophoresed at right angles to the track until a precipitin rocket formed. A second track was overlaid with a suspension of azocoll (5 mg/ml) in 0.05 M Tris-Cl–2 mM $CaCl_2$ (pH 7.8)–1.5% Noble agar and allowed to incubate until digestion of the azocoll could be detected by appearance of red dye in the agar. The third track was stained for protein with a silver stain. A single precipitin rocket was seen at an R_f of 0.54, the azocollagenase clearing zone centered at an R_f of 0.55, and a single silver-stained protein was seen at an R_f of 0.55. Thus, the immunoprecipitin rocket appeared to be due to ISP-1 protein.

To establish that any rockets formed when crude extracts reacted with anti-ISP-1 antibodies were due only to ISP-1 protein, a tandem-crossed immunoelectrophoresis method (16, 24) was used (see above). Precipitin lines of identity (Fig. 3) between the protein in crude cell extracts and the electrophoretically pure ISP-1 suggest that only rockets against ISP-1 were being detected in the crude extracts. When crude cell extract was run alone, only a single rocket was seen with mobility similar to that of ISP-1.

Quantifying ISP-1 protein in crude extracts of *B. subtilis* immunologically. Earlier, Srivastava and Aronson (Fig. 2 in reference 37) showed that 80 to 85% of the soluble azocollagenase present in cell extracts of *B. subtilis* could be attributed to ISP-1. We electrophoresed samples of crude cell extracts from *B. subtilis* 168 cells grown to t_3, $t_{4.5}$, and t_6 in nutrient broth on 7.5% polyacrylamide tube gels; sliced the gels (2-mm thick); and assayed the slices for proteinase activity with azocasein (see above). Recoveries of applied activities were 80 to 100%, and at all times greater than 90% of the azocaseinase activity migrated to a position corresponding to ISP-1 (R_f = 0.65). This result was consistent with findings of Orrego et al. (29) using a different strain of *B. subtilis*.

In addition, we measured the sensitivity of the azocollagenase activity in crude cell extracts prepared at t_4, t_6, and t_8 toward phenylmethylsulfonyl fluoride (2.5 mM) and ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (10 mM), which are inhibitors of ISP-1 (38). Both compounds caused 95 to 99% inhibition in all three samples, again suggesting that ISP-1 is the major azocollagenase activity present at these stages of sporulation.

We wanted next to determine whether the large increases in proteinase activity in cells of *B. subtilis* undergoing sporulation were due to synthesis of new protein or activation of protease already present in the cells. To determine the amount of ISP-1 antigen produced in *B. subtilis* cells during sporulation, we carried out rocket immunoelectro-

phoresis on the soluble fraction of crude cell extracts prepared at different times during the sporulation in nutrient broth (see above). The increases in ISP-1 antigen were compared with the amount of azocollagenase activity in these extracts measured at the same times (Fig. 4A). In the absence of chloramphenicol, the increase in the specific activity of azocollagenase during the period examined was more than 20-fold greater than the increase in the specific activity of the ISP-1 antigen (Fig. 4B). The addition of chloramphenicol not only stopped protein synthesis (appearance of antigen), as expected, but also stopped the activation process.

Previously, we isolated a pleiotropic mutant, *B. subtilis* 168 S-87, which apparently had reduced levels of ISP-1 (8) and carried out the degradation of bulk protein degradation at a reduced rate (21). We examined the levels of ISP-1 antigen in this strain during growth in nutrient broth (Table 1). Although extracts of S-87 contain threefold less total azocollagenase activity than the parental strain, they contain at least as much antigen as extracts of the parental strain (Table 1). In this connection, Neway and Switzer (28) found that strain S-87 contained no collagenase activity eluting from an ion-exchange column in the fractions expected to contain ISP-1 activity.

When cells of the parent strain were grown in the CDSM medium instead of nutrient broth, a marked activation in ISP-1 during the course of sporulation was again observed (Table 1). In fact, the degree of activation of azocollagenase between t_0 and t_8 was 56-fold, which is even greater than the 20-fold seen in nutrient broth-grown cells (Fig. 4B); the specific activity of ISP-1 in *B. subtilis* cells was comparable in the two media.

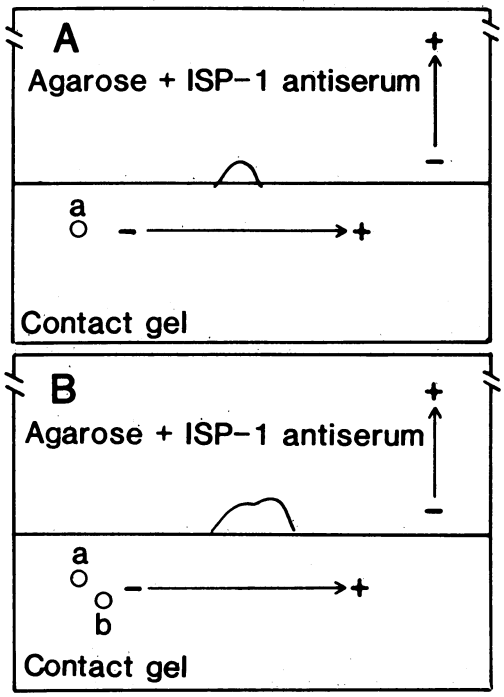

FIG. 3. Traces of tandem-crossed immunoelectrophoresis of ISP-1 and crude cell extracts of *B. subtilis* cells. About 10 μl of ISP-1 recovered from two cycles of affinity chromatography and about 80 μg of dialyzed cell extract of *B. subtilis* cells harvested from nutrient broth-grown cells at t_3 were applied to wells a and b, respectively. Agarose contact gels were electrophoresed until bromphenol dye reached the cathode end. Upper agarose gels containing 100 μl of unfractionated anti-ISP-1 antiserum–10 ml of Tris-Tricine buffer (pH 8.8) were cast adjacent to the contact gels. Electrophoresis was continued at right angles to the first direction. The gels were stained and dried (see the text). Immunoprecipitin lines were traced onto tracing paper. (A) ISP-1 alone (a); (B) ISP-1 (a) in tandem with cell extract (b).

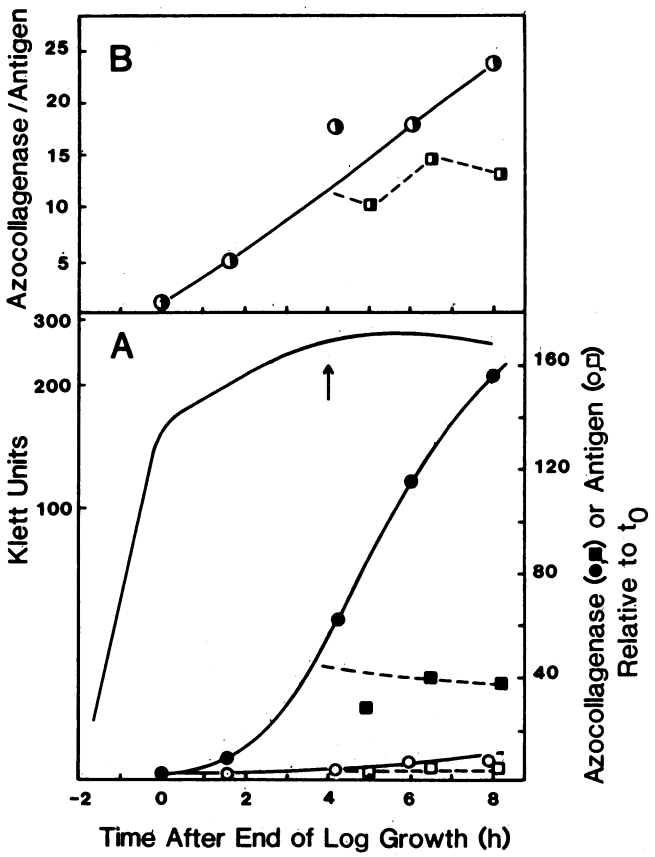

FIG. 4. Time course of azocollagenase and ISP-1 antigen in *B. subtilis* cells during sporulation. (A) *B. subtilis* 168 (Trp^-) cells were grown in nutrient broth, harvested at the indicated times, washed, broken in a French pressure cell, and centrifuged. The clarified supernatant fractions were assayed for azocollagenase and protein and subjected to rocket immunoelectrophoresis. At t_4, one culture was treated with chloramphenicol (100 μg/ml of culture). Azocollagenase units are ΔA_{520}/min mg and antigen units are from measurement of rocket heights and are expressed as mm/mg (see the text). (B) Data is a replot of data from Panel A and units are ΔA_{520}/min per mm for both control culture (solid line) and chloramphenicol-treated (dashed line) cultures. The data shown are from one experiment; duplicate cultures gave similar results.

Presence of an endogenous ISP-1 inhibitor. Millet and Gregoire (26) have previously reported that *B. subtilis* cells contain a heat-stable protein inhibitor of ISP-1 which declines in activity during sporulation. Since loss of such an inhibitor could explain the activations of ISP-1 reported here, we wanted to confirm its presence in these studies. Crude extracts of *B. subtilis* cells (see above) harvested at t_8 were titrated with various amounts of crude cell extracts (unheated) of cells harvested at $t_{1.5}$. A dose-dependent inhibition was observed. When 100 μg of protein from the $t_{1.5}$ extract was added to 1.87 mg of the t_8 extract, the azocollagenase in the latter was inhibited by 50%. Thus, the

TABLE 1. Relative amounts of intracellular proteolytic activity and ISP-1 antigen in *B. subtilis* cells

Strain	Medium	Stage of sporulation	Azocollagenase activity (U/mg)	Antigen concn (mm/mg)	Azocollagenase activity/antigen concn (U/mm, $\times 10^{-3}$)
168 (parental)	HNSM[a]	t_5	0.13	40	3.0
168 (parental)	HNSM	t_8	0.23	57	4.0
S-87	HNSM	t_5	0.062	68	0.9
S-87	HNSM	t_8	0.078	48	1.6
168 (parental)	CDSM	t_0	0.00076[b]	6[c]	0.1
168 (parental)	CDSM	t_3	0.040	37	1.1
168 (parental)	CDSM	t_6	0.28[d]	78[d]	2.6
168 (parental)	CDSM	t_8	0.32	57	5.6

[a] HNSM, Hanson nutrient sporulation medium (10).
[b] Averages of two determinations each of duplicate cultures.
[c] Averages of three determinations each of duplicate cultures.
[d] One culture had anomalously high levels of both antigen and azocollagenase activity.

strain of *B. subtilis* used in these studies also appears to contain an endogenous inhibitor of ISP-1.

DISCUSSION

During sporulation, *B. subtilis* cells appear to degrade bulk protein in a remarkably similar fashion, whether they are developing in nutrient broth or CDSM (compare Fig. 1A to B). This observation suggests that the degradation process is a programmed event of sporulation, which is relatively insensitive to the external medium, provided that nutrient components essential for the sporulation process are present. Furthermore, the exogenous amino acids and peptides present in nutrient broth do not cause the induction of ISP-1.

Earlier Vasantha and Freese (42) found that in cells induced to sporulate by addition of the drug decoyinine (a GMP synthetase inhibitor), ISP-1 activity did not increase measurably until after intracellular proteolysis was well begun, suggesting that under these conditions ISP-1 may not be the rate-limiting step in proteolysis. The data in Fig. 1B strongly suggest that the level of total proteinase activity is not the rate-limiting step in protein degradation during sporulation because, during periods when the intracellular protease activity was dramatically increasing, protein degradation clearly remained a first-order process (for example, between 10 and 13 h of growth; Fig. 1B), instead of becoming a second-order process. Earlier reports of the occurrence of intracellular protease activities and their involvement in protein degradation (4, 19) and sporulation (17, 43), in which unwashed cells were used, must be interpreted very cautiously. Maurizi and Switzer presented evidence (22) that if cells of *B. subtilis* were not properly washed before breaking, that the main proteinase detected in extracts was due to extracellular proteinases associated with the pellet. In this regard, it has been shown recently that neither of the two major extracellular proteases are required for sporulation of *B. subtilis* cells (14, 45). In this study (Fig. 1B), we washed cells using conditions shown previously (8) to eliminate detectable extracellular proteases from the extracts.

Millet and Gregoire (26) reported a low-molecular-weight (M_r = 15,500) protein inhibitor of ISP-1, which disappeared from cell extracts beginning at t_2. If their observations were of any physiological significance, one would predict that ISP-1 should become activated during this period. Based on results of quantitative rocket immunoelectrophoresis, this study has revealed a marked activation of ISP-1 during sporulation. We also showed that some de novo synthesis of ISP-1 occurs during sporulation, which is consistent with an earlier conclusion of Shaginyan et al. (33), which was based only on qualitative immunodiffusion studies. We found 6- to 10-fold increases in antigen concentration, depending on the medium used. Shaginyan et al. (33) reported that titration of ISP-1 with the ISP-1 inhibitor did not alter the immunoprecipitation of ISP-1. In any case, the presence of inhibitor would be expected to mask rather than enhance rocket formation. We conclude that ISP-1 activity in *B. subtilis* cells undergoing sporulation is regulated both by synthesis and posttranslational activation, with the latter being the more important control.

Chloramphenicol had the expected effect (Fig. 4) of stopping increases in ISP-1 antigen concentrations; why it also blocked activation of ISP-1 remains unexplained. It is possible that the synthesis of some protein product may be required to reverse the effects of the ISP-1 inhibitor. For example, Shimizu et al. have recently proposed that a large-molecular-weight membrane proteinase (34) is specifically involved in the inactivation-degradation of the ISP-1 inhibitor (35). Alternatively, the inhibitor protein may be sequestered in the cell or excreted into the medium during sporulation.

It was surprising that cells of *B. subtilis* 168 S-87 (Table 1), which have considerably less total intracellular protease activity than those of the parental strain, contained as much or more ISP-1 antigen. These observations suggest that this mutant may have an altered gene for ISP-1, an aberrant (tightly binding) ISP-1 inhibitor, or elevated levels of the inhibitor. In this regard, Neway and Switzer (28) were unable to elute proteinase activity from an ion-exchange column in the expected position for ISP-1 when cell extracts of strain S-87 were purified by the standard procedure.

The data in Table 1 also show that the activation of ISP-1 is at least as great in cells grown in CDSM as those grown in nutrient broth. If the ISP-1 inhibitor, or degraded fragments thereof, are excreted during sporulation of *B. subtilis* cells, the use of this defined medium may make its detection easier than it would be in a complex medium.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant 5R01-GM19643 from the National Institutes of Health and by grant SO6RR-08136 from the Minority Biomedical Research Support Program.

Justin Neway made useful suggestions to us regarding the use of gramicidin columns.

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