Characterization of Bacillus subtilis Mutants with a Temperature-Sensitive Intracellular Protease

K. J. SASTRY,1 O. P. SRIVASTAVA,2 JAQUELINE MILLET,3 PHILLIP C. FITZJAMES,4 AND ARTHUR I. ARONSON*

Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550;1 Department of Ophthalmology, University of Missouri, Columbus, Missouri 65212;2 Department de Biochimie et Genetique Moleculaire, Institut Pasteur, 75274 Paris, France3; Department of Bacteriology and Biochemistry, University of Western Ontario Medical School, London, Ontario N6A 5C1, Canada4; and Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received 6 July 1982/Accepted 22 September 1982

A colony screening procedure was devised to detect Bacillus subtilis mutants containing temperature-sensitive trypsin-like intracellular protease activity. The enzyme was characterized as a non-sulphydryl serine protease on the basis of inhibitor studies. It was also inhibited by D- or L-histidine but not by any other amino acid tested. The long-term survival at 45°C of these mutants in a minimal salts medium was decreased, with rapid lysis occurring within 24 h. A D-histidine function in long-term survival and inhibition accounted for the presence of additional protease mutants among survivors of histidine auxotrophs selected for their ability to utilize D-histidine. In addition to being lysed when incubated at 45°C under nongrowth conditions, all of the protease mutants had a decreased rate of protein turnover and produced spores deficient in a major low-molecular-weight spore coat polypeptide. The morphology of the undercoat layers was altered, but there was no effect on spore heat resistance or on germination. The missing spore coat polypeptide appeared to be processed from a larger precursor by cleavage to produce N-terminal histidine. A defect in this protease could account for the lack of processing and thus the absence of this polypeptide in spore coats.

Sporulating Bacillus subtilis cells elaborate a variety of intracellular proteases (4, 5, 8, 19). The latter are on the whole dispensable for sporulation (16), whereas the intracellular enzymes seem to play more or less essential roles (8, 11). In an earlier study (8), alteration of the regulatory or structural genes of protease-deficient mutants was not defined. More recently (11), a mutant blocked at an early stage of sporulation was found to have temperature-sensitive protease activity, implying an essential role for this enzyme in sporulation.

Some indication of the functions of intracellular proteases in sporulating bacilli came from a study of a Bacillus cereus mutant producing a temperature-sensitive postexponential protease (3). This mutant was defective in protein turnover and in the processing of a spore coat precursor. As a result, coat-defective spores were formed at the nonpermissive temperature. In addition, there are spore-associated proteases that are very specific for a group of acid-soluble proteins that are rapidly degraded after spore germination (28).

To date, at least two major intracellular proteases have been characterized in sporulating cells of B. subtilis: a major serine protease of the subtilisin type (protease I) (20, 24) and a less prevalent trypsin-like activity (protease II) (9, 23). The latter was unusual in that histidine peptide bonds as well as those containing lysine or arginine were hydrolyzed. The more specific functions of this trypsin-like protease in growth and sporulation were studied by isolating mutants with temperature-sensitive activity. Although no changes in growth rate were found, several postexponential functions were altered, including long-term survival of cells, protein turnover rates, and the structure of the spore coat.

MATERIALS AND METHODS

Isolation of mutants and growth conditions. Protease mutants were isolated from B. subtilis 168 or from this strain obtained from J. Hoch, Scripps Research Institute, designated JH642 (Table 1). Cells were grown and spores were formed in nongrowth conditions. The spores were purified as previously described (18) and then treated with ethyl methane sulfonate (10). After recovery, cells were grown on NSM agar for 16 h at 37°C to provide 1 to 200 colonies per plate. The colonies were replicated onto sterile Whatman 42 disks (9 cm), and the disks were trans-
ferred to petri dishes containing 1.5 ml of 30 mM Tris-
hydrochloride (pH 7.8) plus 150 μg of lysozyme. After
1 h at 37°C, the disks were blotted and transferred to a
second petri dish containing 1.5 ml of 30 mM Tris-
hydrochloride (pH 7.0) plus 4 mM CaCl2 and 2 mM O-
phenanthroline. The dishes were placed at 45°C for 30
min, and then each disk was blotted. The dishes were
then covered with α-N-benzoyl-DL-arginine-p-nitroan-
ilide (BAPNA) at 1 mg/ml (from a stock solution of 40
mg/ml in dimethyl sulfoxide) in 50 mM Tris-hydrochlor-
ide–2 mM CaCl2 (pH 7.8). The disks were incubated
for 2 h at 37°C, and then 1 ml of 0.5% Garett BNC salts
(Sigma Chemical Co.) in 0.2 M Tris-hydrochloride (pH
7.8) was added. Colonies that remained yellowish were
presumed to be mutants and were rescreened as
described above. Those again failing to hydrolyze the
substrate were grown in 100-ml quantities for a direct
 assay of heat-sensitive protease in crude extracts. Two
mutants, designated 1RC and 14AA, were selected for
further study.

Glutamine auxotrophs DRD29 and DRD30 were
selected on the basis of their ability to utilize α-
histidine as a histidine source, with strain DRD27, a
histidine auxotroph, used as the parental strain. These
three strains have been described elsewhere (4).

A mutant, designated B49, that was totally devoid of
protease II activity was isolated after UV treatment
(10−3 survival rate) of strain 168. A colony screening
procedure (13) similar to that described above but
employing α-N-benzoyl-DL-arginine-naphthylamide
(BANA) as substrate was used (2).

**Physiological studies.** Protease II was assayed and
partially purified as described elsewhere (23). The
selectivity of the assay depended on the inclusion of p-
chloromercuribenzoate to inhibit protease I (23) and
the use of BAPNA as a substrate. Either partially
purified or crude extracts were heated at 45°C. Sam-
pled were dissolved in 1 ml of 0.2 N NaOH. One half
was removed for protein determination (12); the other
half was reincubated with an equal volume of 20%
trichloroacetic acid, and the precipitates were collected
on Whatman glass-fiber filters (934AH). The filters
were dried under heat and counted in Omnifluor
cocktail (New England Nuclear Corp.) in an Isocap
300 scintillation counter.

Long-term survival was determined by growing cells
in NSM at 37 or 45°C for 6 to 8 h (end of exponential
growth), centrifuging and washing them once, and
suspending them in the original volume of prewarmed
minimal salts medium (22) including required amino
acids at 50 μg/ml each but lacking a carbon source.
The flasks were reincubated at the previous tempera-
tures, and optical density changes were followed for 24
to 36 h in a Klett colorimeter with a 660-nm filter.
Samples were periodically removed for plating in
duplicate on NSM to determine changes in the viable
number of cells.

**Characterization of spores and spore components.**
Spores were formed in NSM cultures incubated at
37°C, and the spores were purified as previously
described (18). Spores were heat shocked at 70°C for
30 min, and germination studies were done in Pennas-
say broth (Difco Laboratories) or in 50 mM Tris-
hydrochloride (pH 7.6) plus 0.1 M KCl and 10 mM L-
alanine. Optical density changes at 650 nm were
followed in a GCA/McPherson spectrophotometer.

Purified spores were broken by treatment with glass
beads in a Braun disintegrator as previously described,
and spore coats were prepared from the insoluble
fraction by washing (18). Spore coat polypeptides
were solubilized in UDS (8 M urea–1% sodium
dodecyl sulfate–5 mM cyclohexylaminoethanesulfonic
acid–50 mM dithioerythritol [pH 9.8]). The extracts
were fractionated on 5 to 20% gradient acrylamide gels
and stained as previously described (1, 18).

N-terminal amino acids present in the spore coat
proteins or in protease II digests of hemoglobin
were determined by a manual dansylation procedure
(7), followed by hydrolysis in 6 N HCl (27 h at 110°C)
and resolution of dansylated derivatives on two-dimen-
sional polyamide-coated plates (27).

Western blotting of cell extracts fractionated on 5 to
20% acrylamide gradient gels was done as described
elsewhere (26). Cells of strains DRD27 and DRD30
were grown in NSM at 37 or 45°C until various stages
of sporulation (monitored in the phase microscope)
had occurred. Portions (30 ml) were removed at the times indicated in the legend to Fig. 5, centrifuged, washed twice with antibody buffer (50 mM sodium phosphate, 2 mM phenylmethylsulfonyl fluoride, 50 mM NaCl, 0.05% sodium deoxycholate, 5 mM EDTA [pH 8.0]), and lysed by being resuspended in 50 µl of antibody buffer plus 100 µg of lysozyme. After incubation at 37°C for 10 min, the suspension was sonicated for 2 min with a Branson microtip. The extract was centrifuged in a microfuge for 7 min, and the pellet was extracted with UDS by resuspension in 20 µl, boiling for 2 min, and incubation at 37°C for 45 min. After centrifugation in the microfuge for 5 min, the supernatant was pooled with the original extract, and portions were mixed with an equal volume of 40% glycerol-0.05% bromphenol blue for electrophoresis in 5 to 20% gradient polyacrylamide gels at 35 V for 16 h, with buffer constantly recirculated by a peristaltic pump. After being transferred to nitrocellulose and incubated with antibody against the 13,000-dalton spore coat protein, the blots were treated with 125I-labeled protein A and then exposed to X-ray film (X-OMAT AR-2) for varying periods.

**Electron microscopy.** Spore samples for thin sectioning were harvested from NSG agar plates and fixed by a triple fixation method (25). Dehydration and embedding of the samples in Vestopal (Polysciences Corp.) were followed by sectioning and staining by procedures already detailed (5).

**RESULTS**

Several presumptive protease mutants were isolated in three separate screenings of ethyl methane sulfonate-treated spores, with BAPNA used as a substrate. Some were found to contain temperature-sensitive protease II activity in crude extracts, and two, 14AA and 1RC, were selected for further study. The protease II activity in these two mutants was inactivated by heating, as is shown for mutants DRD29 and DRD30 (Fig. 1). One mutant, B49, was found after UV treatment of cells and screening with BANA as substrate. This mutant was devoid of BANase activity when extracts were prepared from cells grown at 37 or 45°C. In all cases, the specific activity of protease I in the mutants (measured with azocoll as substrate) was close to that of the parental strains, and the activity was as heat stable as that of the wild-type protease I.

Initial efforts to map the altered protease marker involved transduction into several auxotrophs, including a glutamine auxotroph, DRD30 (4). Fortuitously, it was found that this particular auxotroph already contained temperaturesensitive protease II activity, in contrast to the parental strain, DRD27 (Fig. 1). Strain DRD30 differed from other glutamine auxotrophs in that it was selected on the basis of its ability to utilize D-histidine (4). This selection involved plating DRD27, a histidine auxotroph, on minimal medium containing 20 µg of D-histidine per ml. Small colonies appearing after 5 to 7 days were screened for a glutamine requirement. Two of three such auxotrophs, DRD29 and DRD30, were found to contain temperature-sensitive protease II (Fig. 1), and thus a basis for this protease alteration was sought.

Protease II in either crude or partially purified extracts was inhibited by D- or L-histidine but by no other amino acid tested (Table 2). It was suspected that this enzyme had a role in protein synthesis.

**TABLE 2. Effect of amino acids on protease II activity**

<table>
<thead>
<tr>
<th>Amino acid added</th>
<th>Sp act (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.20</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>0.50</td>
</tr>
<tr>
<td>D-Histidine</td>
<td>0.55</td>
</tr>
<tr>
<td>L-Proline</td>
<td>1.20</td>
</tr>
<tr>
<td>D-Proline</td>
<td>1.25</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>1.30</td>
</tr>
<tr>
<td>D-Tryptophan</td>
<td>1.25</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>1.30</td>
</tr>
<tr>
<td>D-Tyrosine</td>
<td>1.25</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>1.90</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>1.25</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>1.25</td>
</tr>
</tbody>
</table>

_a_ A crude extract in Tris-CaCl2-p-chloromercuribenzoate was used for the assay of protease II (23). No amino acid inhibited protease I activity (assay of a crude extract with azocoll as substrate). There was more extensive inhibition (85 to 95%) of a purified preparation of protease II by D- or L-histidine, but not by the other amino acids.

_b_ A 20-mM concentration of each amino acid was added to the extract for 10 min at 37°C before the addition of BAPNA.

_c_ One unit is defined as the production of 1 µmol of _p_-nitroaniline at 37°C in 1 min (23).
turnover in nongrowing cells, and indeed the rate in protease II mutants at 45°C was decreased (Fig. 2). The same decreased rates of turnover were obtained with strain B49 at either 37 or 45°C and with strain 14AA at 45°C. It was hypothesized, therefore, that such turnover (or perhaps another function of protease II) was essential for the long-term survival of cells, such as histidine auxotrophs plated on minimal medium plus D-histidine. The ultimate ability to utilize D-histidine (5 to 7 days for colonies to appear) would therefore depend on a functional protease II. It was indeed found that the long-term survival of strain DRD30 at 45°C was much lower than that of the parental strain, DRD27, or DRD30 grown at 37°C (Fig. 3). Similar survival curves were obtained with mutants B49, 14AA, and 1RC. Apparently, inhibition of protease II by D-histidine resulted in lethality for most cells; survival was enhanced by an alteration of protease II that permitted continued function in the presence of D-histidine and thus the selection of double mutants: those with a defective protease II and a glutamine requirement. The latter is related to D-histidine utilization, as described elsewhere (4), and the low frequency of appearance of such mutants (less than 1 in $10^{10}$) is consistent with the isolation of double mutants.

Another possible function of postexponential proteases is in sporulation, such as the processing of spore structural protein precursors (1, 3). The sporulation frequency in NSM of mutants DRD30, 14AA, and 1RC at 45°C was only 70 to 80% of that at 37°C, but the spores were equally heat resistant (100% survival after heating at 70°C for 30 min). Spores of all of the protease II

![Graph](image-url)

**FIG. 2.** Protein turnover in sporulating cultures of *B. subtilis* DRD27 (●) and DRD30 (○). Cells were grown at 37 (A) or 45°C (B) in NSM until 2 h after the end of exponential growth. L-[U-14C]leucine was added to 0.02 μCi/ml for 1 h, and then unlabeled L-leucine was added to 1 mg/ml. Samples (0.5 ml) were removed at the times indicated to measure protein content and trichloroacetic acid-insoluble radioactivity. Values were corrected to a constant amount of protein.

![Graph](image-url)

**FIG. 3.** Optical density changes of strains DRD27 (●) and DRD30 (○). Cells were washed and resuspended in minimal salts medium. Cells growing in NSM at 37 (A) or 45°C (B) were washed and resuspended in minimal salts medium containing essential amino acids at 50 μg/ml. The cultures were reincubated at the same temperatures, and the optical density was measured at the times indicated. There were no significant changes in viable counts for strain DRD27. The viable count for strain DRD30 decreased in parallel with the optical density changes.
mutants formed at 45°C germinated as well as did spores of the parental strain, but they were lacking one of the major low-molecular-weight spore coat polypeptides (Fig. 4). This protein of 12,000 to 13,000 daltons contained N-terminal histidine (17; unpublished data). Protease II is a trypsin-like enzyme that can hydrolyze hemoglobin to produce peptides containing N-terminal histidine residues (data not shown). It was therefore possible that the lack of this spore coat protein was due to the lack or inaccurate processing of a precursor at 45°C. Extracts of sporulating cultures of strains DRD27 and DRD30 were fractionated by gel electrophoresis. After being transferred to nitrocellulose (26), the blots were incubated with antibody against the 13,000-dalton *B. subtilis* spore coat protein and 125I-labeled protein A (Fig. 5). Both strains contained two antigenic components of about 30,000 and 50,000 daltons that cross-reacted with antibody to the 13,000-dalton spore coat polypeptide. In addition, lower-molecular-weight antigens of about 20,000 and 13,000 daltons were present in extracts of DRD27 grown at 37 or 45°C (Fig. 5A and B) and in extracts of DRD30 grown at 37°C (Fig. 5C), but were much reduced in extracts of DRD30 grown at 45°C (Fig. 5D). Similar patterns were obtained when cells were harvested either 2.5 or 4 h after the end of exponential growth. The lack of low-molecular-weight antigens (presumably derived from the larger species) was consistent with a defect in processing of a spore coat precursor.

Resting spores of mutant 14AA formed at 45°C differed in their coat structure from those formed at 37°C. Whereas the latter approached the control (*B. subtilis* JH642) coat in both density of the outer layer and definition of the undercoat striae (Fig. 6A and B), those formed at 45°C showed a thinner, less dense outer coat and a poorly defined undercoat almost totally lacking the usual multilayered appearance (Fig. 6C). The lower density and thinning of the outer coat seen in the mutant at the nonpermissive temperature also occurred in wild-type spores formed at 45°C (Fig. 6D). Thus, the deposition of material in the outer coat was temperature sensitive and correlated with a deficiency of the spore coat protein of 30,000 to 40,000 daltons in spores formed at 45°C (Fig. 4). The defect of undercoat definition seen in the mutants at 45°C was not found in the wild type. This temperature-sensitive lesion appeared to be characteristic of the mutants.

Since autogermation during preparation and fixation markedly enhanced the stainability of the undercoat layers of *B. subtilis* spores, we sought the rare autogermated spore in these preparations. Spores of mutant 14AA formed at 37°C showed undercoat definition of four to five layers (Fig. 7B) almost as clear as those of the wild-type coat (Fig. 7A, also more heavily

FIG. 4. Gel electrophoretic fractionation of extracts of spore coats on a 5 to 20% gradient acrylamide gel. Spores were formed in NSM at 37 or 45°C and purified, and coats were prepared and extracted as described in the text. About 50 μg of protein from each extract was loaded on the gel, electrophoresed at 25 mA for 16 h, and stained. Arrow, Polypeptide of about 13,000 daltons. Strains 14AA and 1RC are protease II mutants; Wt (wild type) and 16UE are the parental strains (JH642). BSA, Bovine serum albumin.

FIG. 5. Autoradiogram of extracts of strains DRD27 grown at 37 (A) or 45°C (B) and DRD30 grown at 37°C (C) or 45°C (D) in NSM until 3.5 h after the end of exponential growth (early stage IV in greater than 80% of the cells). Cell extracts were prepared, and equivalent amounts (ca. 200 μg of protein each) were fractionated by gel electrophoresis and then transferred to nitrocellulose as described in the text. The nitrocellulose was incubated with antibody to the 13,000-dalton spore coat protein and then with 125I-labeled protein A. Arrow, Antigen of 12,000 to 13,000 daltons, the size of the polypeptide in mature spore coats. The size of the other antigens is not accurately known, but all are less than 65,000 daltons (see the text).
FIG. 6. Sections showing the coat region of resting spores of \textit{B. subtilis} strains JH642 and 14AA. (A) Coat of wild-type spore formed at 37°C, showing the dense outer coat (OC) closely applied to the multilayered undercoat (UC) covering the cortical (CX) region. (B) Mutant strain 14AA, at the permissive temperature (37°C), formed an outer coat as dense and robust as that of the wild-type spore and an undercoat which contained multilayers that were somewhat less distinct than those of the wild type. (C) At 45°C, the outer coat of 14AA was narrower and less dense and the undercoat was almost devoid of the striated structure. (D) At 45°C, the coat of \textit{B. subtilis} JH642 also showed the decrease in outer coat deposition shown by 14AA at 45°C, but not the loss in undercoat definition. Bars, 100 nm.
FIG. 7. Sections of spore segments after autogermination, which permits some enhancement of undercoat structure. (A) Wild-type spores of *B. subtilis* JH642 formed at 37°C. (B) Mutant strain 14AA spores formed at 37°C. The undercoat layers are now more distinct where sectioned at right angles. (C) Mutant 14AA spores formed at 45°C. Even where sectioned clearly, the undercoat (UC) shows little or no enhancement of the striated regions. (D) *B. subtilis* JH642 spores formed at 45°C, showing a robust undercoat but poorly deposited outer coat not unlike that of strain 14AA (cf. part C). Bars, 100 nm.
stained). The mutant spores formed at 45°C showed little enhancement of undercoat structure after autogermination. Segments of faint layering were occasionally encountered in an ill-defined undercoat layer (Fig. 7C). The thin, poorly impregnated outer coat but well-defined undercoat seen in sections of wild-type resting spores formed at 45°C were also present when these had autogerminated (Fig. 7D).

DISCUSSION

Several independently isolated protease II mutants had similar phenotypic properties, i.e., decreased rate of protein turnover, more rapid loss of viability under starvation conditions, and lack of a major low-molecular-weight spore coat protein. Most of the mutants were formed after colonies were screened with either BAPNA or BANA as substrate. Two were fortuitously found among D-histidine utilizers. Attempts to map the locus were hindered by lack of rapid, accurate screening for a defective protease. There was always residual protease II activity after colonies were heated to 45°C, so many false positives were found in the colony replica assay. In one series of transduction experiments, phage PBS1 grown on strain JH642 was transduced into strain DRD30, and glutamine prototrophs were selected; 3 to 4% of these contained heat-stable protease II activity. These transductants had regained the parental phenotype for the properties mentioned above, implying that all of the alterations were due to a defective protease II. Five to ten percent of thyA" transformants with DNA from strain B49 were also lacking protease II activity, so it is likely that the protease gene is located in the glnA-thyA region of the B. subtilis chromosome. Further mapping experiments are necessary to define the structural gene and to determine whether mutant B49, totally devoid of protease activity, is a structural or regulatory mutant. The limited mapping data and the independent isolation of several protease II mutants with similar phenotypic properties indicated that all of the alterations detected were due to a defective protease II.

In B. cereus, a mutant with an altered intracellular serine protease had a decreased rate of protein turnover and defectively processed a major spore coat precursor (3). The precursor was normally processed to 13,000-dalton polypeptides that were the major components of the spore coat (1, 3). These were not produced in the mutant at the nonpermissive temperature, and thus coat-deficient spores were formed (3). In the present study, protease II mutants were also defective in turnover and processing, but only for one of several low-molecular-weight spore coat proteins. The Western blots used for the results shown in Fig. 5 were washed free of protein A and antibody with urea-mercaptoethanol and then treated with antibody against a unique 12,000-dalton spore coat polypeptide (6). A major antigen species of about 32,000 daltons was found in extracts of both strains grown at either 37 or 45°C; i.e., there was no detectable alteration in the synthesis or processing of this coat polypeptide. These defective spores were as heat and lysozyme resistant as the wild type and was and germinated at the same rate in either L-alanine plus KCl or Penassay broth. The morphology of the inner spore coat structure of the protease mutants was perturbed (as shown for strain 14AA in Fig. 6B, 6C, 7B, and 7C), presumably owing to a deficiency of the 13,000-dalton polypeptide, although other alterations due to the defective protease cannot be ruled out.

Other B. subtilis mutants containing defective intracellular protease activity were asporogenous (8, 11). It is likely in those cases that another protease, probably protease I, was altered or absent. This protease may have an essential function(s) in the early stages of sporulation as well as an involvement in the formation of structural components, such as the processing of other spore coat proteins. In addition to differing patterns of inhibition (23), only protease I is sensitive to a protein protease inhibitor produced in sporulating cells of B. subtilis (15; unpublished data). The selectivity of this inhibitor implies that regulation of the function of protease I is critical to the sporulation process. A further analysis of mutants containing altered activity should help to resolve the function of these proteases and would also be useful in uncovering any other postexponential, intracellular activities.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM20606 from the National Institutes of Health and funds from the University of Paris VII.

The assistance of Doryth Loewy for the electron microscope work is appreciated.

LITERATURE CITED