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Construction and Properties of an Intracellular Serine Protease Mutant of *Bacillus subtilis*

LOUISE BAND,[1] DENNIS J. HENNER,[1] AND MARK RUPPEN[2]*

Department of Cell Genetics, Genentech, Inc.,[1] and Department of Fermentation Development, Genencor, Inc.,[2] South San Francisco, California 94080

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An intracellular serine protease (ISP-1) mutant of *Bacillus subtilis* was created by introducing a frameshift into the coding region of the cloned gene. Intracellular protease activity in the mutant was very low, yet sporulation in both nutrient broth and minimal medium was normal. The rate of bulk protein turnover in the mutant was slightly slower than that in the wild-type strain. These results suggest that the gene for ISP-1 is not essential and that ISP-1 is not the major enzyme involved in protein turnover during sporulation.

During sporulation, *Bacillus subtilis* produces several intracellular and extracellular proteases. Although the function of these proteases in sporulation is not well understood, it is clear that the alkaline and neutral extracellular proteases are not required for sporulation (14, 18). Little is known about the functions or regulation of the intracellular proteases. It has been suggested that the intracellular proteases function in several important, if not essential, sporulation-associated processes, including enzyme modification and inactivation, bulk protein turnover, and precursor processing (9, 16). There is evidence that one of the intracellular proteases, a trypsinlike serine protease (ISP-2), is involved in protein turnover and spore coat protein precursor processing (11).

The majority of the intracellular protease activity has been attributed to an intracellular serine protease (ISP-1) that has been purified and characterized biochemically (10, 15). Genetic and physiological evidence also indicates that ISP-1 may be involved in protein turnover and may be essential for sporulation. However, these mutant strains had pleiotropic defects, and a comprehensive characterization of the mutation was not presented (5, 7). Cloning the gene and creating a mutation in vitro can eliminate any ambiguity as to the nature of the mutation. Recently, the description of the gene for ISP-1 and its nucleotide sequence were published (8). We also cloned the ISP-1 gene, and in this paper we describe the properties of a strain that lacks ISP-1 activity.

The *B. subtilis* strains and plasmids used in this study are described in Table 1. In order to clone the ISP-1 gene, a series of oligonucleotide probes were designed to complement a portion of the ISP-1 N-terminal amino acid sequence described by Strongin et al. (15). These labeled probes were then used in hybridization experiments designed to detect the gene for ISP-1. While this work was in progress, the cloning and the nucleotide sequence of the gene for ISP-1 was reported (8; T. Beppu et al., Abstr. 3rd Int. Conf. Genetics Biotechnol. Bacilli, 1985). Therefore, based on the reported restriction map and sequence of the gene for ISP-1, the gene *isp* was isolated from a plasmid bank in pJH101 (3) by using a 21-mer oligonucleotide probe (5'ATGAATG GTGAAATCCGCTTG) under high-stringency hybridization conditions (13, 17). A plasmid, designated pISP-2, was isolated that had the expected 3.1-kilobase *Bam*HI fragment with a restriction map as previously identified for the gene *isp* (8).

A mutation was created in the gene *isp* by inserting 4 base pairs at the *Sal*I site in the coding region. This insertion resulted in a frameshift at codon 145 and also brought into frame a termination codon at position 181. This mutation was designated *isp-1*, and a plasmid, pISP-1, carrying the mutated gene for ISP-1 was transformed into *B. subtilis* 168. Replacement of the wild-type gene with the mutant gene required a double crossover event between homologous chromosomal regions and a linear concatemer of plasmid DNA to obtain an *isp-1* strain (14, 19). ISP-1 mutants were identified by assaying extracts prepared from stationary-phase cells grown in nutrient broth. A strain deficient in ISP-1 activity was identified and designated BG3064. Spontaneous chloramphenicol-sensitive clones of BG3064 were isolated, and one, designated BG3036, was chosen for further study (Koide et al. [8] have referred to this same strain as I168 ISPΔ15).

DNA blot analysis of both strains BG3064 and BG3036 revealed a missing *Sal*I site in the coding region of the gene for ISP-1, as expected for the introduction of the *isp-1* mutation (Fig. 1). By using the integrative plasmid JH101 to facilitate mapping, the *isp* gene was mapped by PBS-1 transduction to the *metC* (95% cotransduction)-*argC* (5% cotransduction) region, as has been reported by Koide et al. (8).

When the parent strain 168 was grown in nutrient broth medium, intracellular protease activity was detected in cell extracts at approximately t_3 (Fig. 2). At this time, the enzyme activity increased at least 30-fold, from less than 5 U/ml of culture to 150 U/ml of culture. When BG3036 (*isp-1*) was grown in the same medium, there was less than 5 U of protease activity per ml of culture at the later stages of sporulation. These results not only demonstrate that BG3036 lacks ISP-1 activity but also indicate that ISP-1 accounts for the majority of the intracellular protease activity in sporulating cells, at least when Azocoll is used as the substrate.

In nutrient broth medium, sporulation is initiated by glucose exhaustion (t_0). In strain 168, refractile spores were first observed at t_7, and heat resistance developed at t_8. Generally, at t_{20}, 60 to 80% of the cells present were heat-resistant spores. In the ISP-1 mutant, the frequency of sporulation was the same as that in the wild type. Sporulation of BG3036 was also normal in S-7 minimal medium (4).

* Corresponding author.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or feature	Source
168	*trpC*	J. Hoch
BG212	*metC argC purB*	*Bacillus* Genetic Stock Center
BG3064	*trpC2 isp-1*::pISP-1	This study
BG3065	*trpC2 isp*::pISP-2	This study
BG3036	*trpC isp-1*	This study
pJH101	Cm^r Ap^r	J. Hoch
pISP-2	ISP (wild type) in pJH101	This study
pISP-1	ISP (mutant) in pJH101	This study

Although the frequencies of sporulation for both the parent (0.45%) and the mutant (0.52%) were lower in S-7 minimal medium than in nutrient broth, both strains sporulated at the same frequency. Sporulation of both strains was also examined in another well-defined minimal medium that gave a higher frequency of sporulation, CDSM medium (6). In CDSM medium, sporulation of strains 168 and BG3036 was essentially identical (J. Hageman, personal communication). These observations contradict the results that had been previously reported (8) and suggest that ISP-1 is not required in either complex or minimal medium as a scavenging enzyme functioning to supply the cell with amino acids derived from protein degradation.

In strain 168, the rate of protein turnover increased during sporulation to 13% turnover per h, whereas in strain BG3036 the rate increased to 10% turnover per h (Fig. 3). Repeated

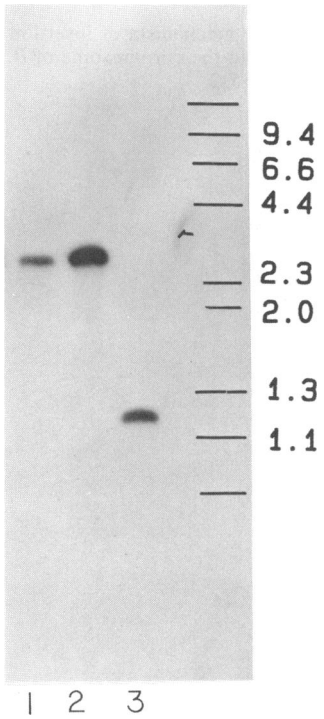

FIG. 1. DNA blot analysis of the ISP-1 mutant genes. Chromosomal DNA digested with *Bam*HI and *Sal*I was separated on an agarose gel. After transfer to nitrocellulose, the DNA was probed with the 1-kilobase *Bam*HI-*Hin*dIII fragment of pISP-1. Lanes: 1, BG3036; 2, BG3064; 3, 168 (wild type). Size standards (in kilobases) are indicated to the right.

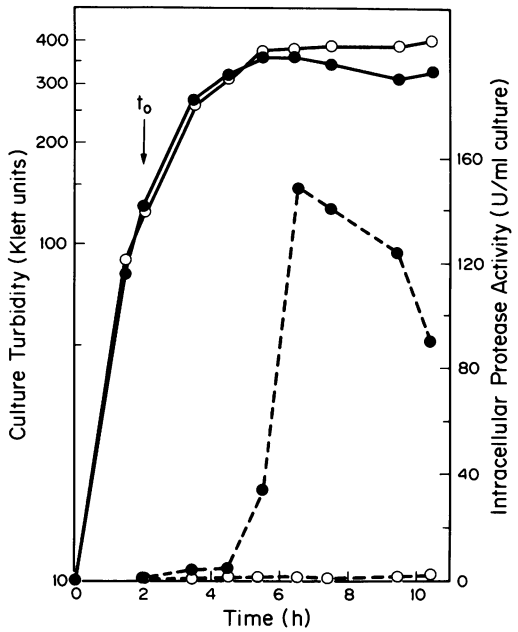

FIG. 2. Intracellular protease activity during growth and sporulation. Strains 168 and BG3036 were grown in nutrient broth plus 0.1% glucose (12). Samples were removed, and intracellular protease activity was determined by using Azocoll as the substrate (2). The end of exponential growth, t_0, was at 2 h. Symbols: ●, strain 168; ○, strain BG3036; ——, growth (Klett units); – – –, protease activity. One unit of protease activity is defined as the digestion of 1 μg of Azocoll per min per ml of culture.

experiments consistently showed that the rate of turnover in BG3036 was slightly less than the rate observed in the wild-type strain. In CDSM medium, the rates of protein degradation were identical in the two strains (data not shown). These results indicate that ISP-1 is not the major enzyme involved in bulk protein turnover during sporulation. However, it is not clear whether the pattern of protein degradation in the mutant is qualitatively the same as that in the wild type. The small difference in bulk protein turnover rates (13 versus 10% turnover per h) might represent the lack of degradation of a small family of proteins. Bond et al. (1)

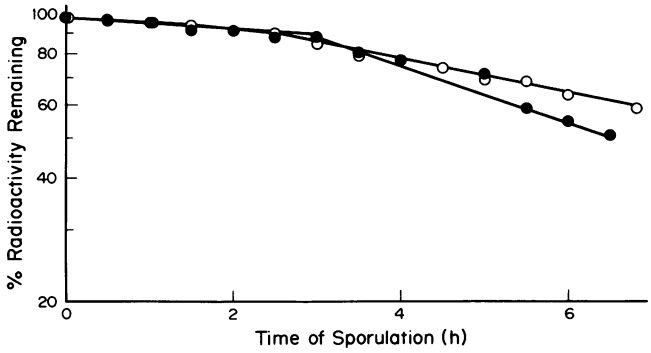

FIG. 3. Protein turnover during sporulation. Strains 168 and BG3036 were labeled with [^{3}H]leucine and grown in nutrient broth plus 0.1% glucose essentially as described by Bond et al. (1). This method of determining protein turnover involved measuring the release of acid-soluble radioactivity. The value for each point was defined as 100 − the percent trichloroacetic acid soluble radioactivity for that sample. Symbols: ●, strain 168; ○, strain BG3036.

have shown that the stability of a small family of specific proteins is regulated by nutritional conditions during exponential growth of *B. subtilis* (1). A similar situation might exist in sporulating cells in which ISP-1 is responsible for the degradation of a small group of specific proteins. Other proteases might be able to compensate for the loss of ISP-1 activity and allow turnover to occur at a normal rate. Although the function of ISP-1 in sporulation and turnover has not been determined, it is clear that it is not required for normal sporulation.

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