Characterization of the Gene Encoding an Intracellular Proteinase Inhibitor of *Bacillus subtilis* and Its Role in Regulation of the Major Intracellular Proteinase

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The gene (ipi) for an intracellular proteinase inhibitor (BsuPI) from *Bacillus subtilis* was cloned and found to encode a polypeptide consisting of 119 amino acids with no cysteine residues. The deduced amino acid sequence contained the N-terminal amino acid sequence of the inhibitor, which was chemically determined previously, and showed no significant homology to any other proteinase inhibitors. Analysis of the transcription initiation site and mRNA showed that the *ipi* gene formed an operon with an upstream open reading frame with an unknown function. The transcriptional control of *ipi* gene expression was demonstrated by Northern (RNA) blot analysis, and the time course of transcriptional enhancement roughly corresponded to the results observed at the protein level. Strains in which the *ipi* gene was disrupted or in which BsuPI was overexpressed constitutively sporulated normally. Analysis of the time course of production of the intracellular proteinase and proteinase inhibitor in these strains suggested that BsuPI directly regulated the major intracellular proteinase (ISP-1) activity in vivo.

*Bacillus subtilis* cells produce extracellularly at least four serine proteinases (Apr, Epr, Bpf, and Vpr) and three metalloproteinases (NprA, NprB, and Mpr), all of which have been shown to be nonessential for normal growth and sporulation (15, 44, 50). As for intracellular proteinases, four serine proteinases, ISP-1, ISP-2 (generally considered to be a trypsin-like proteinase), ISP-3, and a membrane-bound proteinase (29, 41–43, 46), and a spore-germinating proteinase (Gpr) (48) are known. Among them, ISP-1 is the major intracellular proteinase, accounting for 80% of the intracellular azocollagen or azocasein hydrolytic activity, and is the most well characterized (5). ISP-1 was thought to function in the degradation and turnover of bulk proteins during sporulation (5). However, the gene encoding ISP-1 has already been cloned (17) and a mutant deficient in it was shown to sporulate normally (1). Expression of the ISP-1 gene was shown to be regulated at the transcriptional level indirectly by Spo0A and various regulatory proteins (16, 34). Transcription was enhanced at *Tm*, the earliest stage of sporulation, but ISP-1 activity was not detected in cells until about 4 h later (34). Although the N-terminal 17-amino-acid sequence of the primary translational product was not found in purified ISP-1, it was reported that this cleavage was an artifact and not related to the activation of ISP-1 (39). These results suggested that ISP-1 activity was masked posttranslationally and revealed when needed.

On the other hand, ISP-1 was found to be inhibited by an intracellular proteinase inhibitor of *B. subtilis* (26). This inhibitor (designated BsuPI, in accordance with our proposal [40]) was purified by two different groups (27, 31) and analyzed at the protein level (30). Although there are some differences between the results obtained by the groups, BsuPI was reported to be a very thermostable protein with a molecular mass of about 16 kDa and the decrease in its cellular amount seemed to cause an increase in ISP-1 activity (27, 42). Nishino and Murao reported that BsuPI strongly inhibited ISP-1 activity and was inactivated by a membrane-bound proteinase under acidic conditions when tested in vitro (30). These results suggested that BsuPI inhibited ISP-1 activity through complex formation at an early stage of sporulation and that later BsuPI was inactivated by a membrane-bound proteinase, resulting in high ISP-1 activity. Therefore, for characterization of the exact function of BsuPI with special reference to sporulation, cloning of the BsuPI-encoding gene was deemed to be essential.

In addition, it was also reported that BsuPI contained no cysteine residues. Since the structural relationship between the disulfide bridge and the reactive site in serine proteinase inhibitors from eukaryotic organisms (20, 33) and *Streptomyces* species (28) is already well known, it would be interesting to determine how BsuPI inhibits ISP-1. In this report, cloning of the BsuPI-encoding gene and characterization of its structure and function are described.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, media, and transformation.** *Escherichia coli* XL-1-blue (Stratagene) and BL21(DE3) (Novagen) were grown at 37°C in 2YT and M9 (37) or M9ZB (47), respectively, and then transformed by the method of Hanahan (13). *B. subtilis* DB104 (15) was grown at 37°C in L broth (37) or 2× SG (21) and then transformed by the method of Chang and Cohen (7) or Sadaia and Kada (35). When required, ampicillin, kanamycin, and erythromycin were added at concentrations of 50, 5, and 10 µg/ml, respectively. For molecular cloning, pBR322 (37) and its derivative pBR-AN3 (49) were used, and for sequencing, M13mp18 and M13mp19 (37) were used. For gene expression in *E. coli*, pET-11d (47) was used. In the gene disruption experiment, plasmid pUC118 (37) was used as the vector. *spo0A* mutants UOT-1611 (spo0AΔHB trpC2 lys-1 nprE18 nprR2 aprEΔ3), whose *spo0A* mutation is particularly stable, and UOT-1277 (spo0A12 trpC2 lys-1 nprE18 nprR2 aprEΔ3) were kindly supplied by Y. Kobayashi and F. Kawamura, respectively.

**Preparation of DNA.** Chromosomal DNA of *B. subtilis* was prepared by the method of Saito and Miura (36). Large- and

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Multiprime DNA labeling system (Amersham) and used as a probe. DNA sequencing was performed with an M13 Sequencing Kit, Version 2.0 (Takara Shuzo Co.). We named the BsulI-encoding gene *ipi* (a gene coding for intracellular proteinase inhibitor).

Expression of the BsulI-encoding gene in *E. coli* and *B. subtilis*. An expression vector for BsulI was constructed as follows. The 1.4-kb HindIII-PstI fragment (Fig. 1) of the BsulI-encoding gene was shortened with exonuclease BAL 31 from the HindIII site to the PstI site and treated with the Klenow fragment of DNA polymerase I in the presence of all four deoxynucleotides triphosphates. An XbaI linker was ligated to the fragment and then digested with both XbaI and PstI. The digested fragments were inserted into pUC119 and sequenced. Among them, a 1.1-kb fragment truncated up to nucleotide position 1,109 (see Fig. 2), which contained the putative ribosomal binding site and the translational initiation codon for BsulI, was obtained. This DNA fragment, DH5, was used for expression of BsulI. For expression in *E. coli*, the DH5 fragment was inserted between the XbaI and HindIII sites of pET-11d, and the resultant plasmid, pET-DH5, was introduced into *E. coli* BL21(DE3).

For expression in *B. subtilis*, the DH5 fragment was ligated with the 0.4-kb Sau3AI-Alul fragment of pUB110, which contained the promoter of the kanamycin resistance gene, and then introduced into the pPviII site of pUB110. *B. subtilis* DB104 was transformed with this plasmid (pUB-DH5).

**Purification of BsulI expressed in *E. coli***. *E. coli* BL21(DE3) harboring pET-DH5 was cultured in 1 liter of M9ZB medium for 3.5 h, and then transcription from the T7 promoter was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (47). Incubation was continued for a further 4.5 h. Cells were harvested by centrifugation (6,000 × g, 10 min, 4°C) and dissolved in 25 ml of distilled water. The cell suspension was then passed through a French pressure cell, and cell debris was removed by centrifugation (25,000 × g, 10 min, 4°C). The supernatant was heated at 70°C for 10 min, and then the denatured proteins were removed by centrifugation. Then 1 N HCl was added to adjust the pH to the supernatant to 4.0. After incubation at 37°C for 30 min, the denatured proteins were removed as described above. The pH of the solution was adjusted to 7.0 with 1 N NaOH, and then solid ammonium sulfate was added to 80% saturation. The precipitate obtained on centrifugation was dissolved in a minimum volume of buffer A (20 mM Tris-HCl buffer, pH 7.5) and then dialyzed against the same buffer overnight at 4°C. After removal of insoluble materials by centrifugation, the dialysate was applied to a DEAE-cellulose column (2.6 by 3.5 cm) preequilibrated with buffer A. Elution was performed with an NaCl gradient (0 to 0.5 M) in buffer A, and BsulI was eluted in the 0.22 M fraction. The BsulI-containing fractions were then combined and dialyzed against buffer B (20 mM triethylamine, pH 7.3). After removal of the insoluble materials, the dialysate was applied to a Mono-Q HR5/5 column (Pharmacia) preequilibrated with buffer B. Elution was performed with a NaCl gradient (0 to 0.35 M) in buffer B. BsulI was eluted in the 0.2 M fraction and detected as a single band on SDS–15% polyacrylamide gel electrophoresis (PAGE) (19), which was followed by staining with Coomassie brilliant blue.

**Determination of the N-terminal amino acid sequence of recombinant BsulI**. The N-terminal amino acid sequence of purified recombinant BsulI was determined with a gas phase protein sequencer (ABI 470-120A). The sample for sequence analysis was prepared by the method of Matsudaira (22).

**Analysis of BsulI mRNA**. Total RNA of *B. subtilis* cells was prepared by the hot-phenol method (45) with the modifica-
B. subtilis was cultured in 500 ml of L broth in a 2-liter flask at 37°C, and at various phases of growth, 50 ml of the culture was removed, mixed with 0.5 ml of ice-cold 1 M NaNO₃–0.1 M MgCl₂, and then rapidly chilled on ice-water. Cells were harvested by centrifugation (6,000 × g, 10 min, 4°C) and then suspended in 5 ml of ice-cold TES (30 mM Tris-HCl buffer [pH 8.0], 5 mM EDTA [pH 8.0], 50 mM NaCl) containing 25% sucrose and 3 mg of lysozyme per ml. Incubation on ice-water was continued for 15 min. After centrifugation (3,500 × g, 20 min, 4°C), the spheroplast pellet was resuspended in 3 ml of 0.02 M sodium acetate (pH 5.5)–0.5% SDS–1 mM EDTA, and then hot phenol extraction was performed three times. After ethanol precipitation, the pellet was dissolved in 100 μl of diethylpyrocarbonate-treated distilled water and then kept at −80°C until use.

Northern (RNA) blot analysis was performed as described previously (37). A 30-μg sample of total RNA prepared from cells at various phases of growth was subjected to electrophoresis on 1% agarose containing formaldehyde and then blotted onto a Hybond-N+ membrane (Amersham). Hybridization was performed at 65°C overnight in 10 ml of 0.5% SDS–5× SSC–5× Denhardt’s solution containing 50 μg of sonicated salmon sperm DNA per ml with the multiprime-labeled 1.3-kb BamHI-SphI fragment as a probe (2.0 × 10⁶ cpmp). The membrane was washed twice with 0.2× SSC–0.1% SDS at 65°C for 20 min each time.

Primer extension analysis was performed as described by Débarbouille and Raibaud (10). A deoxynucleotide nucleotide with the sequence 5′-TCATGTGGCGATGTGAAGT-3′, which is complementary to the sequence from nucleotides 821 to 840 (see Fig. 2), was synthesized at the Center for Gene Research of Nagoya University and then used as a primer. A 100-μg sample of total RNA extracted from Tₘ (1.5 h after Tₘ) cells was hybridized with 50 ng of primer at 30°C overnight and used for the assay. The length of the primer-extended product was determined by comparison with sequencing ladders generated by using the same primer and the single-stranded DNA template containing the BsuPI-encoding gene ranging from nucleotides 339 to 2260 (1.9-kb PstI fragment).

Construction of an in-frame lacZ fusion vector as a promoter probe. The promoterless lacZ gene was obtained from pMC1405 (6), digested with both BamHI and DraI, and then inserted into BamHI-SmaI-digested pUC18 (pUClacZ). From the BsuPI-encoding gene and its flanking region, a 0.9-kb PstI-HindIII fragment for an in-frame ipa-lacZ fusion and a 1.0-kb HindIII-BamHI fragment for orf1-lacZ were prepared, treated with T4 polymerase in the presence of all four deoxyneclosephosphate triphosphates, and then inserted into the 5′-upstream region of the lacZ gene in pUClacZ. In-frame fusions were selected on the basis of the formation of blue colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-containing plates and confirmed by nucleotide sequencing. The fusion genes thus constructed were isolated and inserted into appropriate sites of pUB110, to which the multicloning site region of pNU210 (49) was incorporated, in the direction avoiding readthrough from any promoters of the plasmid (24) and then introduced into B. subtilis. β-Galactosidase activity, due to the promoter activity of the inserted fragment, in B. subtilis was judged on the basis of the formation of blue colonies on X-Gal plates, and the activity in liquid culture was measured by the method of Miller (25).

Disruption of the BsuPI-encoding gene by homologous recombination. pUB110 was partially digested with HaeII, and the 1.0-kb DNA fragment containing the whole kanamycin resistance gene was recovered from the agarose gel after electrophoresis. The 1.0-kb HindIII-SphI fragment of the BsuPI-encoding gene (Fig. 1) was inserted between the same restriction sites of pUC118, and then the kanamycin resistance gene was introduced into the NspV site of the BsuPI-encoding gene of the plasmid. A 1.2-kb ClaI-SacI fragment containing the erythromycin resistance gene obtained from pHW1 (14) was introduced into the SacI-AecI site of this plasmid (pYEK13). B. subtilis was then transformed with pYEK13 by the method of Sadaie and Kada (35), and kanamycin-resistant and erythromycin-sensitive colonies were selected. ChromosomalDNAs of these clones, which were expected to be recombinants on both side of the disrupted BsuPI-encoding gene on pYEK13, were extracted, and insertion of the kanamycin resistance-encoding gene into the chromosomal BsuPI-encoding gene was confirmed by Southern blot analysis. Among the clones with disrupted BsuPI gene, number 42 (designated 42pi) was chosen for further analysis.

Spatial disruption of BsuPI-defective and overproducing strains. B. subtilis DB104, 42pi, and DB104 (pUB-DHS) were inoculated into 5 ml of 2× SG medium at an initial A₆₀₀ of 0.08 and then incubated for 24 h at 37°C with shaking. Heat-resistant spores were counted by plating cells on tryptose blood agar base supplemented with 0.5% glucose after heating the culture for 10 min at 80°C. Strains 42pi and DB104 (pUB-DHS), on the other hand, were replicated onto L agar containing kanamycin to confirm maintenance of the kanamycin resistance gene.

Determination of intracellular proteinase activity. B. subtilis DB104, 42pi, and DB104 (pUB-DHS) were inoculated into 500 ml of 2× SG medium and then cultured at 37°C. At various phases of growth, 5 ml of the culture was removed and cells were harvested by centrifugation (3,500 × g, 10 min, 4°C). Cell extracts were obtained as described by Ruppen et al. (34).

Intracellular proteinase activity was determined as described by Chavira et al. (8). One proteinase unit was defined as the amount of proteinase that hydrolyzed 1 μg of azocollagen (Azocoll; Sigma) per min.

Intracellular proteinase inhibitor activity was determined as described previously (40). Samples were prepared after heat treatment at 95°C for 10 min and removal of denatured proteins by centrifugation. Proteinase activity was then measured as described above. One inhibitor unit was defined as the amount of inhibitor that caused a 50% reduction of azocollagenase activity (2 U) of subtilisin Carlsberg (Sigma) under the assay conditions used. Subtilisin was used as the proteinase for the assay because it was quantitatively inhibited by BsuPI (31).

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank nucleotide sequence data bases under accession number D16311.

RESULTS

Cloning and sequencing of the BsuPI-encoding gene, ipi. An oligonucleotide probe corresponding to the N-terminal amino acid sequence of BsuPI was used to determine that the gene for BsuPI is located on a 1.9-kb PstI fragment as described in Materials and Methods. The 1.9-kb PstI fragments were isolated, inserted into the PstI site of pBR-AN3, and used to transform E. coli. Colony hybridization with the above-described probe identified four positive colonies containing a 1.9-kb PstI fragment among approximately 1,000 ampicillin-resistant colonies. Sequence analysis revealed that this fragment contained three open reading frames (ORFs), and the longest one was cloned incompletely. Therefore, the 1.3-kb HindIII fragment containing the 5′-upstream region of this ORF was also cloned and sequenced. These ORFs were 840,
228, and 357 nucleotides long, respectively. The second ORF (ORF2) was contained within the first ORF (ORF1), in a different reading frame. The deduced amino acid sequence of the product of the third ORF matched that of BsuPI, as reported by Nishino et al. (31), indicating that ORF3 encodes BsuPI (Fig. 1 and 2).

The deduced amino acid sequence of BsuPI contained no cysteine residues, as reported previously (31). Although it was reported that BsuPI contained no histidine residues (31), the deduced amino acid sequence of BsuPI contained one histidine residue. Putative ribosomal binding sites complementary to the 3′ sequence of the 16S rRNA of *B. subtilis* (3) were found just upstream of the putative translation initiation codons (ATG for ORF1 and the BsuPI-encoding gene and TGG for ORF2) of these ORFs, although that of ORF2 was less matched. An 11-bp-long palindromic sequence (AG = −108 kJ/mol), followed by a T stretch, was found downstream of the BsuPI-encoding gene and may function as a ρ-independent transcriptional terminator. However, no palindromic sequence was found in the region between ORF1 (or ORF2) and the *ipi* gene. Southern blot analysis revealed only one BsuPI-encoding gene in the *B. subtilis* genome (data not shown).

**Production of recombinant BsuPI in *E. coli***. The BsuPI-encoding gene was cloned downstream of an IPTG-inducible T7 promoter in *E. coli* expression vector pET-11d to create plasmid pET-DH5 as described in Materials and Methods. After IPTG induction, the cell lysate of *E. coli* BL21(DE3) harboring pET-DH5 contained significant inhibitory activity toward subtilisin Carlsberg, which the lysate of uninduced cells exhibited no activity. The recombinant protein was soluble and had a molecular mass of 16 kDa, corresponding to the mass of BsuPI estimated by SDS-PAGE. This protein, purified as a single band on SDS-PAGE as described in Materials and Methods, retained inhibitory activity against subtilisin. The N-terminal amino acid sequence of the purified inhibitor, MNENQEVVLXI (X = not identified), was determined by Edman degradation and matched that of native BsuPI except for the first methionine, which was absent in the native form (31).

**Northern blot analysis of BsuPI mRNA**. On Northern blot analysis of RNA prepared from *B. subtilis* at various phases of growth (Fig. 3), an RNA of about 0.9 kb was specifically detected with the BsuPI gene probe (1.3-kb BamHI-SphI fragment in Fig. 1) but not with the 5′-upstream probe (1.0-kb HindIII-BamHI fragment) or the 3′-downstream probe (0.4-kb SpII-PstI fragment) (data not shown). As can be seen in Fig. 3, an about 1.6-kb RNA was also detected, but this signal was detected with all of the probes described above (data not shown), suggesting that the band resulted from nonspecific hybridization with the probes. These results also suggest that the whole transcription unit of the BsuPI-encoding gene is contained in the 1.3-kb BamHI-SphI fragment (see below) and the 0.9-kb RNA is the mRNA for BsuPI synthesis. Expression of the BsuPI-encoding gene was enhanced at about T2 and continued to be so to at T5.5 (Fig. 3). The amount of 16S rRNA was also monitored as a control and found to be unchanged throughout T2 to T5.5 (data not shown). The results obtained with the BsuPI mRNA are in general agreement with the results obtained at the protein level (42) up to T5 and suggest that BsuPI expression is controlled mainly at the transcriptional level.

**Determination of the promoter region and transcription initiation site**. To determine the transcription initiation site for the BsuPI-encoding gene, a series of *ipi-lacZ* or orf1-lacZ in-frame fusion plasmids were constructed (Fig. 1) and introduced into *B. subtilis*. Among them, *B. subtilis* harboring pPLL-PNs, pPLL-PHf, and pPLL-BHF formed blue colonies on X-Gal plates but *B. subtilis* harboring pPLL-HdHf did not, even after being cultured for up to 7 days. Furthermore, 3′-promoter activity was completely lost on deletion of the *BamHI-NcoI* fragment or the 170-bp *NcoI*-HindIII fragment. These results indicate that the 0.3-kb *BamHI*-HindIII fragment contains the promoter region and the sequence around the *NcoI* site of this fragment is important for transcription of the BsuPI-encoding gene. pPLL-HdHf did not form a blue colony, suggesting that ORF1 is not transcribed in *B. subtilis*.

Upstream from the BsuPI-encoding gene, several sequences similar to the Sp0A-binding sequence (4) were found (Fig. 2). To examine the role of Sp0A in the expression of the BsuPI-encoding gene, pPLL-PHf was introduced into *sp0A* mutant strains (UOT-1611 and UOT-1277), but these strains showed as high a level of β-galactosidase activity as the wild-type strain, indicating that the promoter was functional in the absence of Sp0A. This showed that Sp0A, which is known to control expression of the ISP-1-encoding gene indirectly (16, 34), is not necessary for expression of the BsuPI-encoding gene and, moreover, σ factors involved downstream of the Sp0A cascade may not be relevant to BsuPI expression (4, 12, 16).

**Primer extension analysis** revealed that transcription of the BsuPI-encoding gene starts at nucleotides 623 and 624, which are about 40 bp upstream of the *NcoI* site described above (Fig. 4). In the region 5′ upstream from this site, a −10 sequence for σ70 (GACCGTAT/com consensus GCCGATAT) was found but a −35 sequence was not. No other promoter-like sequence for σ factors of *B. subtilis* was found (18). To determine whether ORF1 is transcribed, a deoxyoligonucleotide with the sequence 5′-CGCGATGATGACGATGA GTA-3′, complementary to the sequence from nucleotides 274 to 294 (Fig. 2), was used as a primer for reverse transcriptase. However, no primer-extended product was observed. This result suggests that ORF1 is not transcribed under the cultural condition employed.

**Effects of constitutive overexpression and disruption of the BsuPI-encoding gene on sporulation**. Strains containing a disruption of the BsuPI-encoding gene were constructed by insertion of a kanamycin resistance gene. Southern blot analysis was performed on chromosomal DNAs extracted from 12 kanamycin-resistant and erythromycin-sensitive clones, obtained as described in Materials and Methods, by using a 0.4-kb *HindIII*-NspV fragment labelled with 32P as a probe. Among them, 10 had the 1.0-kb insert of the kanamycin resistance gene within the BsuPI-encoding gene, as expected (data not shown).

To determine the relationship of growth phase-specific expression of the BsuPI-encoding gene to sporulation of *B. subtilis*, heat-resistant spores of the wild-type strain, DB104; one of the insertion mutants, 42pi; and a strain overexpressing the BsuPI-encoding gene, DB104(pUB-DH5), were counted as described in Materials and Methods. While the numbers of viable cells of these three strains per milliliter were 9.9 × 109, 2.1 × 109, and 1.1 × 109, respectively, the numbers of spores per milliliter were 5.9 × 1012, 8.0 × 1012, and 3.9 × 1012, respectively. As for *B. subtilis* 42pi and DB104(pUB-DH5), more than 98% of the colonies derived from spores retained the kanamycin resistance gene. Thus, the three strains can sporulate efficiently, indicating that BsuPI is not necessary for sporulation of *B. subtilis*.

**Interaction of BsuPI with intracellular proteinase in vivo**. The time course of the intracellular proteinase and proteinase inhibitor activities was analyzed by using cell extracts of *B. subtilis* DB104, 42pi, and DB104(pUB-DH5). As shown in Fig.
**FIG. 2.** Nucleotide sequence of the gene encoding BsuPI and the deduced amino acid sequences of three ORFs. Only the antisense strand is shown. The vertical arrows above the DNA sequence indicate the positions of the 5' termini of the BsuPI-encoding gene transcripts determined by primer extension analysis (see Fig. 4). The possible σ37-type −10 region is doubly underlined. Putative ribosome-binding sites (S D) are underlined. Putative initiation codons and termination codons are shown in bold letters. An 11-nucleotide palindromic sequence is indicated by horizontal arrows below the DNA sequence. The region considerably homologous to the SpoOA-binding site is indicated by dots above the nucleotide sequence.
5, no proteinase inhibitor activity was detected in the BsuPI-defective strain (42pi). In this strain, proteinase activity was detected earlier than in the parental strain and the timing of its appearance was in agreement with that of ISP-1-encoding gene expression (34). In contrast, proteinase activity was not detected in the BsuPI-constitutive strain. These data demonstrate that BsuPI controls the major intracellular proteinase, ISP-1, in vivo and that no other proteinase inhibitor for ISP-1 exists in B. subtilis.

**DISCUSSION**

By using the cloned BsuPI-encoding gene, we were able to pursue the role of BsuPI during growth and sporulation of *B. subtilis*. The transcriptional control of BsuPI gene expression was demonstrated by Northern blot analysis, and the time course of transcriptional activation roughly corresponded to the results obtained at the protein level (Fig. 3 and 5). Furthermore, recombinant BsuPI with N-terminal methionine exhibited inhibitory activity toward subtilisin Carlsberg. These results demonstrated that BsuPI was active without posttranslational modification, such as removal of the N-terminal methionine and proteolytic processing, as was observed in the case of the proteinase inhibitor of *B. brevis* (40). Therefore, growth phase-specific transcriptional activation of the BsuPI-encoding gene may have some significance at the early stage of sporulation, regardless of its necessity for sporulation. Northern blot analysis also revealed that the BsuPI mRNA has a long 5’-upstream control region and that it might be transcribed as an operon with ORF2 (Fig. 2).

The transcriptional initiation site of the BsuPI-encoding gene was detected by primer extension analysis. Although the fusion assay was performed under multicopy conditions, transcriptional activation with the same timing was also observed on time course analysis of β-galactosidase production in *B. subtilis* harboring pPL-PHf (data not shown). These findings, together with the results obtained with the fusion gene shown in Fig. 1, suggest several possibilities concerning the control of BsuPI expression. For example, the product of ORF2 may be essential for BsuPI-encoding gene expression, which is similar to the finding that genes for a proteinase and its activator protein were in the same operon as reported in *B. stearothermophilus* (32). The positively charged amino acid residues abundant in the ORF2 product might be significant for binding to DNA.

Although only the −10 consensus sequence recognized by σ^P^ in the upstream sequence of the transcriptional initiation site and σ^P^ is presumed to function in vegetative cells (18), a mechanism involving cooperation of σ^P^ and some factor, like that of σ^V^ and SpoOA in sporulating cells, might be necessary for expression of the BsuPI-encoding gene (38). Further study is needed to identify which σ factor recognizes the promoter of the BsuPI-encoding gene.

Time course analysis of production of the intracellular proteinase and proteinase inhibitor in the BsuPI-deficient and -constitutive strains showed that BsuPI strongly inhibited the major proteinase (ISP-1) in vivo at least until T_s, so that a lag between transcription of the ISP-1-encoding gene and the appearance of ISP-1 activity was seen (Fig. 5). However, ISP-1 activity increased at T_a, even during BsuPI-encoding gene transcription (Fig. 3), and an abrupt increase of ISP-1 activity at T_s was observed in the wild-type strain, as well as in the BsuPI-deficient strain (Fig. 5). These results cannot be explained simply by BsuPI inactivation by the membrane-bound proteinase, as was reported previously (30, 42, 43), since the ISP-1 increase was observed even in the BsuPI-deficient strain. The reason for these changes in ISP-1 activity is not clear, but they might have some relevance to the finding that ISP-1 is inhibited less by BsuPI from cells at later stationary phases (39).

The BsuPI-deficient strain and the BsuPI-overexpressing strain sporulated efficiently. This indicates that BsuPI is not
important for normal sporulation. Since an ISP-1-deficient strain also sporulates normally (1), the function of BsuPI was assumed to involve inhibition of ISP-1 activity, which might be harmful only at an early stage of sporulation. It may be noted that with a longer culture period (3 days), the BsuPI-deficient strain tended to lyse more easily than the normal strain in medium containing bonito extract instead of beef extract.

BsuPI was reported to contain no cysteine residues, as judged from amino acid composition analysis. Although the relationship between the disulfide bridge and the reactive site of proteinase inhibitors has been well characterized for eukaryotic inhibitors (20, 33), no such relationship is known for bacterial inhibitors other than those from *Streptomyces* spp.

Recently, proteinase inhibitor genes were cloned from gram-negative bacteria, *E. coli* (9, 23), and *Pseudomonas aeruginosa* (11). Their deduced amino acid sequences contained cysteine residues. We cloned an extracellular inhibitor (BbrPI)-encoding gene from *B. brevis* which also contained no cysteine residues (40). Primary structure comparison seemed to be the first step for identification of the reactive site and characterization of the inhibitory mechanism for these cysteine-less inhibitors from bacilli, but BsuPI shows no homology to other inhibitors or even to BbrPI. This suggests that these inhibitors evolved through different pathways.

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