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Construction of a *Bacillus subtilis* Double Mutant Deficient in Extracellular Alkaline and Neutral Proteases

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A mutant strain of *Bacillus subtilis* carrying lesions in the structural genes for extracellular neutral (*nprE*) and serine (*aprA*) proteases was constructed by the gene conversion technique. This mutant had less than 4% of the extracellular protease activity of the wild type and sporulated normally, indicating that neither of these sporulation-associated proteases is essential for development.

Spore formation in *Bacillus subtilis* is initiated near the end of exponential growth. During sporulation, *B. subtilis* undergoes a sequence of biochemical and morphological changes under genetic control. The appearance of at least two extracellular proteases—a neutral metalloprotease sensitive to EDTA and an alkaline serine protease (subtilisin) sensitive to phenylmethylsulfonyl fluoride—is closely associated with this sequential order of gene expression (3, 9). Mutants deficient in the structural gene of the neutral protease (*nprE*) demonstrate that this enzyme is not essential for sporulation (7, 16). On the other hand, there are conflicting reports on whether alkaline protease plays a role in the normal development of a spore (5, 6, 13).

In this communication, we describe the construction of a double mutant carrying a deletion in the alkaline protease gene (Δ*aprA3*) and an uncharacterized lesion in the neutral protease gene (*nprE18*). The data show that neither extracellular protease is required for normal sporulation in *B. subtilis*.

We have recently reported the cloning, genetic mapping, and sequencing of the S fragment containing the promoter proximal half of the extracellular alkaline protease gene (1, 17). (This gene was previously called *sprE* in reference 17.) As shown in Fig. 1, we constructed plasmid pUBHSΔ3, which deleted a 178-base-pair *Hpa*I fragment encoding part of the leader peptide of alkaline protease. We predicted from the sequence data (17) that this deletion would result in a truncated leader peptide of 25 amino acid residues.

A list of *B. subtilis* strains used is shown in Table 1. To construct a neutral protease- and alkaline protease-deficient double mutant (*nprE18*Δ*aprA3*), we first transferred the *nprE18* and *nprR2* mutations from NT18 to DB100 (NIG1121) and obtained DB102 (*nprE18 nprR2 his*). It has been shown that competent cells of *B. subtilis* have a strong correction system for mismatched paired DNA resulting in gene conversion (4). We therefore next applied the gene conversion technique to transfer the 178-base-pair *Hpa*I deletion (Δ*aprA3*) into the chromosome of a neutral protease-deficient mutant, DB102 (*nprE18 nprR2*). We transformed competent cells of DB102 with pUBHSΔ3 DNA and selected kanamycin-resistant (Km^r) transformants on tryptose-blood agar base (Difco Laboratories) supplemented with 0.5% glucose and 5 μg of kanamycin per ml. We then assayed protease production on Schaeffer sporulation agar plates (12) containing 5 μg of kanamycin per ml and 2% skim milk. Four of 200 Km^r transformants showed no extracellular protease activity and retained the pUBHSΔ3 plasmid, indicating that the frequency of gene conversion was 2%. Two protease-deficient Km^r transformants were cured of the plasmid by allowing them to sporulate in Schaeffer sporulation medium. After incubation at 37°C for 24 h, cells were heated at 80°C for 10 min and plated on tryptose-blood agar base. Km^s cells cured of the plasmid appeared at a frequency of about 2%. Two of these were designated DB104 and DB105 (*nprE18 nprR2 aprA*Δ*3*).

We used Southern blotting (14) to confirm that these strains carried the expected deletion in the S fragment. To visualize the 1.2-kilobase *Hin*dIII (S fragment) and the 1-kilobase *Hin*dIII fragment (Δ*aprA3*), we exposed the nitrocellulose strip containing *Hin*dIII-digested *B. subtilis* chromosomal DNA from DB102, DB104, and DB105 to radioactively labeled M13mp9 containing the S fragment (Fig. 2). The 178-base-pair deletion in the S fragment was introduced into the chromosome of DB102.

Table 2 shows the activities of extracellular protease and the sporulation frequencies of DB101, DB102 (*nprE18 nprR2*), DB104 (*nprE18 nprR2* Δ*aprA3*) and DB105 (*nprE18 nprR2* Δ*aprA3*). Only 2 to 4% of the protease activity of the wild type was found in the supernatants from cultures of DB104 and DB105. As pointed out by Stahl and Ferrari (15), a certain level of protease activity was expected because of cell lysis and release of intracellular proteases during growth and because of the secreted esterase (bacillopeptidase F), which was recently reported by Roitsch and Hageman (10).

TABLE 1. *Bacillus subtilis* strains

Strain	Genotype	Source[a]
NT18	*purB6 metB5 trpB3* Str^r *nprR2 nprE18*	K. Yamane (16)
DB100	*his metB*	Y. Sadaie (11)
DB101	*his*	NT18$^{tf}\rightarrow$DB100
DB102	*his nprR2 nprE18*	NT18$^{c}\rightarrow$DB100
DB103	*his nprR2 nprE18* Δ*aprA3*(pUBHSΔ)3	pUBHSΔ3$^{tf}\rightarrow$DB102
DB104	*his nprR2 nprE18* Δ*aprA3*	
DB105	*his nprR2 nprE18* Δ*aprA3*	

[a] Symbols for genetic crosses are (tf) transformation and (c) transformation with relevant donor marker transferred by congression during selection for unlinked marker.

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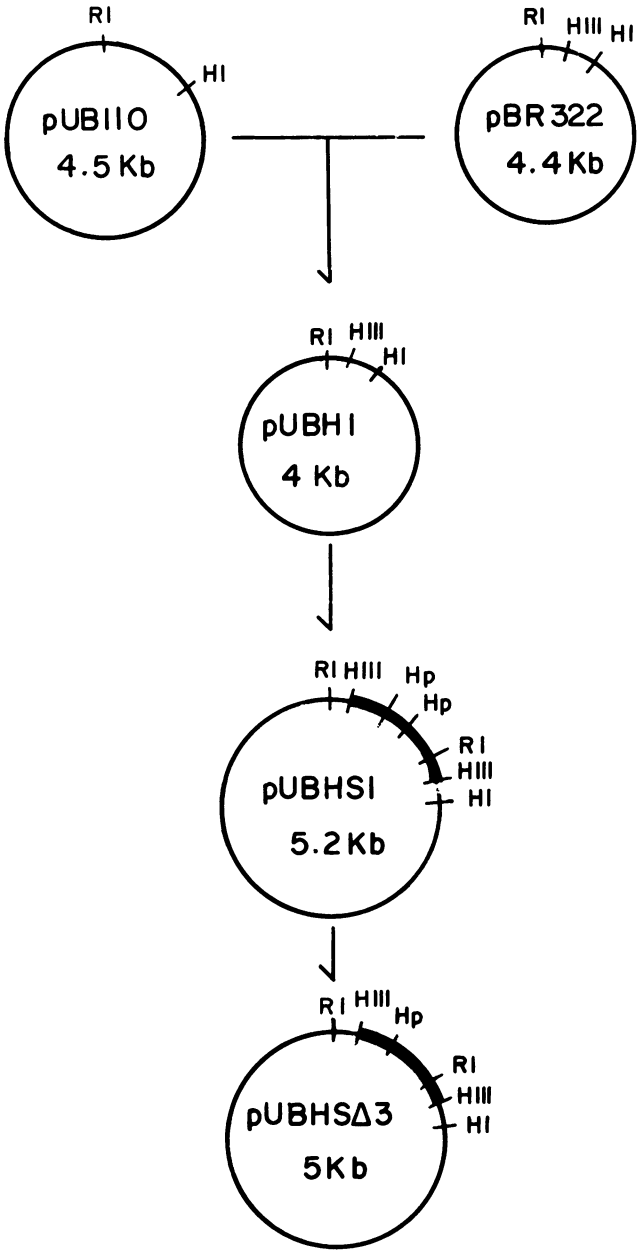

FIG. 1. Construction of recombinant plasmid pUBHSΔ3. The *Eco*RI-*Bam*HI fragment of pUB110 (2) was substituted with the *Eco*RI-*Bam*HI fragment of pBR322 to create a single *Hin*dIII site. From the resultant plasmid pUBH1, pUBHS1, which contained the S fragment, was constructed. pUBHSΔ3, which had a deletion of an 178-base-pair *Hpa*I fragment in the S fragment, was constructed by cutting pUBHS1 with *Hpa*I and ligating with T4 ligase. The thick line indicates the S fragment. RI, HI, HIII, and Hp indicate restriction endonuclease sites for *Eco*RI, *Bam*HI, *Hin*dIII, and *Hpa*I, respectively.

It is evident from Table 2 that both extracellular proteases, neutral protease (*nprE*) and alkaline protease (*aprA*), are not required for normal sporulation in *B. subtilis*.

The extracellular neutral and alkaline protease-deficient mutant described here should be useful for the excretion of useful foreign peptides as well as for the purification of proteins from *B. subtilis*.

TABLE 2. Activities of extracellular proteases and sporulation frequency[a]

Strain	Activity of proteases (%)	Viable cells per ml	Spores per ml
DB101	100	5.3×10^8	4.1×10^8
DB102 (*nprE18*)	65	1.0×10^9	8.8×10^8
DB104 (*nprE18 ΔaprA3*)	2.6	1.0×10^9	7.9×10^8
DB105 (*nprE18 ΔaprA3*)	4.1	9.1×10^8	7.6×10^8

[a] Protease activity in the supernatant of T3 (3 h after end of exponential growth) culture in 2× SG (5) medium was measured by the method of Millet (8). Cells were inoculated into 2× SG medium at an initial absorbancy at 660 nm of 0.08 and incubated for 24 h at 37°C with shaking. Heat-resistant spores were counted by plating the cells on tryptose-blood agar base medium supplemented with 0.5% glucose after heating for cells for 10 min at 80°C.

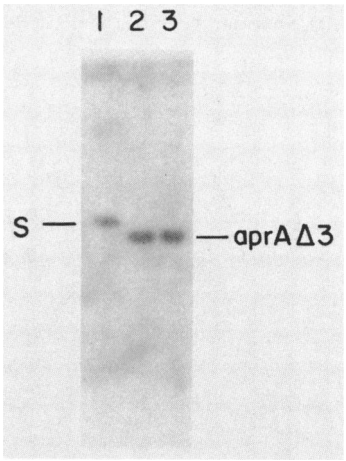

FIG. 2. Southern hybridization of the ^{32}P-labeled S fragment to DB102 (*nprE18*), DB104 (*nprE18 ΔaprA3*), and DB105 (*nprE18 ΔaprA3*) DNA. Each DNA was digested with *Hin*dIII, electrophoresed in 0.8% agarose gel, and blotted onto a nitrocellulose filter as described by Southern (14). The ^{32}P-labeled S fragment was prepared from M13mp9 containing the S fragment. Hybridization was performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate–50% formamide solution at 40°C for 20 h (14). Slots: 1, DB102 DNA; 2, DB104 DNA; 3, DB105 DNA. The S is about 1.2 kilobases (1), and the *ΔaprA3* is about 1 kilobase by deletion and relative mobility on the gel.

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