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Replacement of the *Bacillus subtilis* Subtilisin Structural Gene with an In Vitro-Derived Deletion Mutation

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The entire subtilisin structural gene from *Bacillus subtilis* I168 has been cloned, and its nucleotide sequence has been determined. When expressed on a high-copy-number shuttle vector, a fivefold increase in serine protease activity was observed. The DNA sequence of the gene is 80% homologous to the *Bacillus amyloliquefaciens* subtilisin structural gene, and the translated mature coding sequence is 85% homologous to the published protein sequence of subtilisin BPN'. The chloramphenicol resistance determinant of a plasmid integrated at the subtilisin locus was mapped by PBS1 transduction and was found to be linked to *glyB* (83%) and *argC* (60%), but not with *metC* or *purB*. The chromosomal locus containing the wild-type subtilisin allele was replaced with an in vitro-derived allele of the gene (Δ*apr-684*) that contained a 684-base-pair deletion. The technique used for introducing the deletion is a variation of the gene replacement methods used in *Saccharomyces cerevisiae* and *Escherichia coli*. When used in *B. subtilis*, deletion mutants could be directly screened among the transformants. Physiological characterization of the Δ*apr-684* mutation revealed no discernable effect on the formation of heat-resistant endospores, but strains carrying the mutation produced only 10% of wild-type serine protease activity. A model is presented that outlines the pathway for plasmid integration and deletion formation in *B. subtilis*.

Under conditions of nutrient deprivation, *Bacillus subtilis* I168 initiates a developmental program from which heat-resistant endospores are produced. Closely associated with this temporal and sequential order of gene expression is the appearance of an esterase and at least two proteases in the supernatant of the culture. The two best-characterized proteases are the neutral protease, a metalloenzyme sensitive to EDTA, and an alkaline protease (subtilisin), a serine protease sensitive to phenylmethylsulfonyl fluoride (17, 18, 26, 44).

Considerable controversy has been generated over whether these hydrolytic enzymes have a role in the normal development of the endospore or act simply as scavenging enzymes when usable nutrients have been depleted from the medium (14, 31). Mutations that block sporulation at the earliest stage of development (stage 0 mutations) are pleiotropic and result in the secretion of little or no subtilisin into the culture supernatant. Analysis of mutations in these *spo0* loci has resulted in the suggestion by some investigators that the expression of extracellular subtilisin is required for normal sporulation (14, 31). Many of the subtilisin-deficient mutants that have been isolated are pleiotropic and are blocked at an early stage in sporulation (17, 18). Shoer and Rappaport (39) characterized a *B. subtilis* I168 serine protease mutant and isolated an extracellular protein that was shown to be a fragment of the wild-type enzyme. Further characterization showed only 1 in 53 molecules to be functional, but the strain carrying the mutant allele sporulated normally (39). The chromosomal map location of this mutation was not determined. Mutations have been isolated in the structural gene of the neutral protease (*nprE* locus), and studies have shown that a functional gene product is not required for normal sporulation (26, 44).

In this paper, the structural gene of *Bacillus amyloliquefaciens* subtilisin was used as a hybridization probe to isolate the *B. subtilis* I168 subtilisin gene. The nucleotide sequence of the *B. subtilis* subtilisin gene was determined, and the map location was determined by integrating a chloramphenicol-resistant plasmid at the subtilisin locus. This particular method of mapping cloned genes in *B. subtilis* is a widely accepted technique (9, 13). The chromosomal locus containing the subtilisin gene was then replaced in vivo with an in vitro-derived version of the gene that contained a 684-base-pair (bp) deletion. The technique used has been termed gene replacement and is a variation of methods reported for use in *Saccharomyces cerevisiae* and *Escherichia coli* (12, 36, 40). Physiological characterization of the subtilisin deletion mutation in *B. subtilis* I168 revealed no discernible effect on endospore development. However, strains carrying the deletion mutations produced only 10% of wild-type serine protease activity.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study are listed in Table 1. *E. coli* MM294 was cultured in L broth and transformed by the procedure of Dagert and Ehrlich (6). *B. subtilis* strains were cultured on tryptose-blood agar base (Difco Laboratories) or minimal glucose medium and were transformed by the procedure of Anagnostopoulos and Spizizen (2). The preparation of PBS1 transducing lysates and PBS1 transduction and mapping have been described elsewhere (15). The cloning vector used in this study is pBS42, an *E. coli-B. subtilis* shuttle vector (4). The plasmid consists of the pBR322 origin of replication, the pUB110 origin of replication, and the chloramphenicol acetyl transferase gene from pC194, a *Staphylococcus aureus* plasmid. The plasmid pJH101 (10) was kindly provided by J. Hoch. The plasmid pS4 contained the subtilisin structural gene from *B. amyloliquefaciens* and was kindly provided by J. Wells (46).

Media and reagents. Bacterial alkaline phosphatase, *Kpn*I, and *Hinc*II were from Bethesda Research Laboratories, Inc. *Pvu*I, *Pvu*II, *Eco*RI, *Nco*I, T4 DNA ligase, and the large fragment of DNA polymerase I were from New England

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TABLE 1. Designation, genotype, and origin of strains of *B. subtilis*

Strain	Relevant genotype	Origin
I168	*trpC2*	J. A. Hoch
JH703	*trpC2 pheA12 Δspo0A677*	J. A. Hoch
IA84	*glyB33 metD1*	BGSC[a]
IA151	*argC4 hisA1 phe-1 catA*	BGSC
BG16	*purB6 metB5 leuA8 lys-21 hisA thr-5 sacA321*	A. Galizzi (PB 1665)
BG31	*glyB133 hisH metA29 thi-78 recH342*	Prozorov
BG77	*trpC2 prt-77*	NTG × I168[b]
BG81	*metB5 prt-77*	BG16 DNA × BG77[c]
BG84	*Δspo0A677 prt-77*	JH703 DNA × BG81
BG2014	*trpC2 apr-1*::pIV6	pIV6 DNA × I168[d]
BG2015	*glyB133 hisH metA29 thi-78 recH342 apr-1*::pIV6	pIV6 DNA × BG31
BG2016	*trpC2 prt-77 apr*::pIDV1.4	pIDV1.4 DNA × BG77
BG2017	*trpC2 prt-77 Δapr-684*::pIDV1.4	pIDV1.4 DNA × BG77
BG2018	*trpC2 prt-77 Δapr-684*	Cm[s] of BG2017[e]
BG2019	*Δapr-684*	BG2018/PBS1 × IA84
BG2020	*argC4 hisA1 phe-1 catA apr-1*::pIV6	pIV6 DNA × IA151[f]

[a] Bacillus Genetic Stock Center, Columbus, Ohio.

[b] The protease deficient mutation, *prt-77*, was obtained by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) mutagenesis of strain I168. The phenotype of this mutant is explained in the text.

[c] Strain BG77 was transformed with BG16 DNA.

[d] When the plasmid pIV6 was transformed into strain BG77, a protease-deficient phenotype was observed (data not shown). In theory this plasmid should interrupt the subtilisin-coding sequence upon integration; consequently all strains transformed with this plasmid were assigned a genotypic designation of *apr-1*::pIV6.

[e] A spontaneous chloramphenicol-sensitive derivative of strain BG2017 was isolated by growth of the culture in the absence of antibiotic selection.

[f] Strain IA84 was transduced with a PBS1 lysate made from strain BG2018.

Biolabs. Selection for plasmid transformants in *E. coli* was on LB agar containing 12.5 μg of chloramphenicol per ml. Plasmid transformants of *B. subtilis* were selected on tryptose-blood agar base or LB plus skim milk agar supplemented with 5 μg of chloramphenicol per ml. LB plus skim milk agar contained 1.5% (wt/wt) Carnation powdered nonfat milk. LB plus starch agar contained 1% (wt/wt) soluble starch (Sigma Chemical Co.). Serine protease activity was assayed in solution by measuring the change in adsorbance at 412 nm per minute upon incubation with 0.2 mg of succinyl(-L-Ala-L-Ala-L-Pro-L-Phe)*p*-nitroanilide (Vega) per ml in 0.1 M sodium phosphate (pH 8) at 25°C (7, 46). Cultures were grown to the late-logarithmic phase of growth in modified Schaeffer medium (17); upon continued incubation, samples were removed every 2 h to assay supernatants for subtilisin activity. The percentage of mature endospores was determined from dual platings on tryptose-blood agar base plates before and after heating samples of the culture for 10 min at 80°C. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis was performed by the method of Adelberg et al. (1).

Physical characterization of the cloned DNA. Plasmid DNA was prepared from *E. coli* transformants by the alkaline lysis method of Birnboim and Doly (5). *B. subtilis* chromosomal DNA was prepared by the method of Marmur (22). DNA fragments from restriction enzyme digests were resolved and analyzed by electrophoresis on 1% agarose or 6% polyacrylamide gels. Restriction fragments to be sequenced were ligated into appropriate sites of M13 phage vectors mp8 or mp9 (25). DNA sequencing was carried out by dideoxy methods (35). DNA probes were labeled with [α-^{32}P]CTP by nick translation (32). For Southern hybridization analysis, digested DNA fragments were separated on 1% agarose and depurinated as described by Wahl et al. (45). The DNA was transferred to nitrocellulose by the method of Southern (41). Hybridization and washings were performed as described by Maniatis et al. (20).

RESULTS

Isolation of protease-deficient mutants. To facilitate selection of plasmids containing protease genes, a strain that was deficient in protease production was constructed. Strain I168 was mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and plated on skim milk plates. Colonies producing a smaller halo were picked for further analysis. Each colony was characterized for protease production on skim milk plates and amylase production on starch plates. One such isolate, which was partially protease deficient (Prt$^{+/-}$), amylase positive, and sporulation positive, was designated BG77, and the mutation was designated *prt-77*. The *prt-77* allele was placed in a *spo0A* background by congression (Table 1). This strain, BG84, was completely devoid of protease activity on skim milk plates.

Cloning and expression. Southern blot analysis of *B. subtilis* I168 chromosomal DNA digested with *Eco*RI showed that a single 6-kilobase (kb) *Eco*RI fragment hybridized with a labeled fragment from the *B. amyloliquefaciens* subtilisin gene (46; J. Wells, unpublished results). Slices in the 6-kb range were cut from a preparative agarose gel of *Eco*RI-digested I168 DNA. The DNA was electroeluted and ligated into pBS42 that had been digested with *Eco*RI and treated with bacterial alkaline phosphatase. Amplification of the ligation mixture was accomplished by transforming *E. coli* MM294 and isolating plasmid DNA from a suspension of 5,000 pooled colonies. This plasmid pool was transformed into *B. subtilis* BG84 (Table 1), a protease-deficient strain, and protease overproducing colonies were screened by plating on LB agar plus skim milk. Plasmid DNA was isolated from protease overproducing colonies and examined by Southern analysis for a 6-kb *Eco*RI insert that hybridized to a fragment from the C terminus of the subtilisin structural gene from *B. amyloliquefaciens*. A positive clone was identified by hybridization, and the plasmid was designated pS168.1. *B. subtilis* BG84 transformed with pS168.1 secreted serine protease at a level fivefold over that produced in *B. subtilis* I168. *B. subtilis* BG84 does not produce detectable levels of serine protease when assayed with the specific chromogenic substrate as described above. The addition of EDTA to the supernatants did not affect the assay results, but the addition of phenylmethylsulfonyl fluoride to the supernatants reduced protease activity to undetectable levels.

Physical characterization of the cloned DNA. A partial restriction map of the 6.5-kb *Eco*RI insert is shown in Fig. 1. The subtilisin gene was localized to within the 2.5-kb *Kpn*I-*Eco*RI fragment by subcloning in pBS42 the two *Kpn*I-*Eco*RI fragments and testing for overexpression of subtilisin in *B. subtilis* BG84. Southern hybridization experiments with a labeled fragment from the C terminus of the *B. amyloliquefaciens* subtilisin gene as a probe localized the C terminus of the *B. subtilis* gene to within or part of the 631-bp *Hin*cII fragment B in the right-end *Kpn*I-*Eco*RI fragment (Fig. 1). The tandem *Hin*cII fragments B, C, and D and *Hin*cII-*Eco*RI

fragment E (Fig. 1) were ligated into the appropriate M13 vectors for DNA sequence analysis. The sequence of this region is shown in Fig. 2.

Mapping of the subtilisin structural gene by PBS1 transduction. The 516-bp *Hin*cII fragment C, which contains part of the putative "pro" sequence and approximately 50% of the coding sequence for mature subtilisin (Fig. 1 and 2), was ligated into pJH101 that had been digested with *Hin*cII and treated with bacterial alkaline phosphatase. This plasmid, pIV6, was transformed into *B. subtilis* I168 and strain BG31, and single colony transformants were colony purified twice and used to prepare PBS1 transducing lysates. These strains were designated BG2014 and BG2015, respectively (Table 1). Strains IA84 and IA151 were transduced with a PBS1 lysate made from BG2014, and chloramphenicol resistance was found to be linked to *glyB* (83%) and *argC* (60%). Additional linkage data were accumulated in a three-factor cross, (*glyB*, Cm^r, *metA*), when BG31 was transduced with this same lysate. The results of a five-factor cross with *glyB*, *catA*, *metD*, and *argC* are shown in Table 2. Figure 3 summarizes the linkage data and shows the order to be (*glyB catA*) Cm^r *metD argC metA*.

Isolation of the structural gene deletion mutation. To construct an integration plasmid carrying a defective subtilisin gene with a 684-bp deletion, a two-step ligation was required. These constructions are illustrated in Fig. 4. The plasmid carrying the 6.5-kb insert, pS168.1, was digested with *Eco*RI, and the single-stranded ends were filled in with the appropriate deoxynucleotides by treatment with the Klenow fragment of DNA polymerase I. This DNA was restricted with *Hin*cII, and the 800-bp *Eco*RI-*Hin*cII fragment E (Fig. 1), which contains, in part, the 5′ end of the subtilisin gene, was purified by electroelution from a preparative polyacrylamide gel. This fragment was ligated into pJH101 that had been digested with *Hin*cII. The resultant plasmid, pIDV1, contained fragment E in the orientation shown in Fig. 4. *Hin*cII fragment B of pS168.1, which contains the 3′ end of the subtilisin gene, was gel purified as described above and ligated into pIDV1 that had been digested at the unique *Hin*cII site. Restriction analysis of the resulting plasmids identified one, designated pIDV1.4, that contained fragment B in the correct orientation with respect to fragment E. This plasmid pIDV1.4 contains a deletion derivative of the subtilisin gene and portions of the 5′ and 3′ flanking sequences.

Upon transformation of *B. subtilis* BG77 (*prt-77*, Prt$^{+/-}$) with pIDV1.4, two classes of Cm^r transformants were obtained, 75% showed parental level of proteases (Prt$^{+/-}$), and 25% appeared almost completely protease deficient (Prt$^-$) as observed by zones of clearing on plates containing LB agar plus skim milk. The Cm^r Prt$^-$ class was unexpected and could not be due to a single crossover integration of the plasmid at the homologous regions for fragment E or B because the gene would not be interrupted. In fact, when either fragment E or B was ligated independently into pJH101 and subsequently transformed into *B. subtilis* BG77, the protease-deficient phenotype was not observed (data not shown). The Cm^r phenotype of Cm^r Prt$^-$ pIDV1.4 transformants was found to be unstable, and Cm^s Prt$^-$ derivatives could be isolated at a frequency of about 0.1% after 10 generations of growth in minimal medium in the absence of antibiotic selection.

Chromosomal DNA was obtained from BG77 and the Cm^s Prt$^-$, Cm^r Prt$^-$, and Cm^r Prt$^{+/-}$ derivatives of transformations with pIDV1.4. These chromosomal DNA preparations were digested with *Eco*RI and *Nco*I, electrophoresed on agarose gels, and transferred to nitrocellulose filters. The hybridization pattern of these DNAs, when probed with the labeled 2.5-kb *Kpn*I-*Eco*RI fragment (Fig. 1), is shown in Fig. 5. The structure of the chromosome at the wild-type subtilisin locus is diagramatically shown and confirmed by the hybridization data in lane b. The 1.7-kb *Eco*RI-*Nco*I band contains the 684-bp sequence to be deleted. Lane d is DNA isolated from a Cm^r Prt$^{+/-}$ transformant (BG2016). As seen from data, the structure of this particular Cm^r Prt$^{+/-}$ transformant was derived from a single crossover and integration of the plasmid at the homologous region for fragment E. Lane c contains DNA isolated from a Cm^r Prt$^-$ transformant (BG2017); when the structure is determined from the Southern data, the only difference between the Cm^r Prt$^-$ and Cm^r Prt$^{+/-}$ strains is the 684-bp deletion (fragments C and D) within the subtilisin structural gene. Lane a contains DNA from the Cm^s Prt$^-$ strain (BG2018) and is a Cm^s derivative of BG2017 (lane c). No plasmid DNA remained at the subtilisin locus, but, as expected, the *Eco*RI-*Nco*I fragment is reduced in size from 1.7 kb (lane b) to 1.0 kb (lane a). Other enzymatic digests and Southern blots have confirmed the architecture of these DNAs to be as illustrated in Fig. 5 (data not shown).

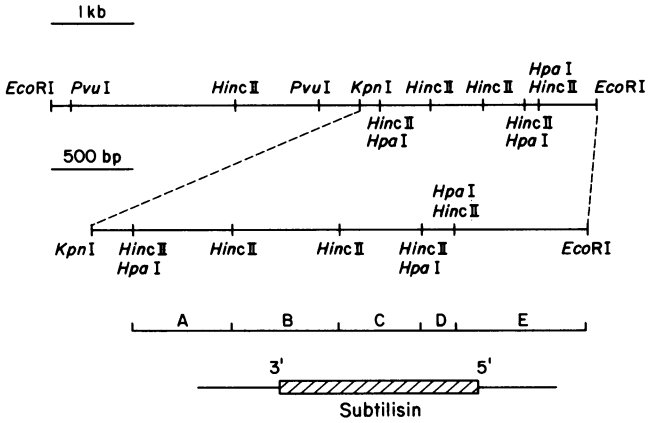

FIG. 1. Partial restriction map of the 6.5-kb *Eco*RI fragment that contains the functional *B. subtilis* I168 subtilisin structural gene. A functional gene is contained within the expanded map of the 2.5-kb *Kpn*I-*Eco*RI subclone. The tandem *Hin*cII fragments and the *Hin*cII-*Eco*RI fragment are functionally labeled with the letters A through E to aid in the description of plasmid constructions and the data presented in Fig. 5 and 6. Fragments C and D are entirely within the proposed translated sequence of subtilisin, whereas fragments B and E contain part of the 3′ and 5′ translated sequence, respectively.

The genotypic designations for the pIDV1.4 transformants and derivatives were assigned as follows. The Cm^s Prt$^-$ phenotype carries no detectable plasmid DNA, but does carry a 684-bp deletion within the subtilisin structural gene (Δ*apr-684*). The Cm^r Prt$^-$ phenotype carries the deletion and pIDV1.4 (Δ*apr-684*::pIDV1.4), whereas the Cm^r Prt$^{+/-}$ strain has no mutation, but carries pIDV1.4 integrated at the *apr* locus (*apr*::pIDV1.4).

Because the exact nature of the *prt-77* mutation in *B. subtilis* BG77 was not fully understood and to simplify interpretations of the protease assay and sporulation data, the Δ*apr-684* allele was transferred to *B. subtilis* IA84 by PBS1 transduction. The *apr* locus maps between *glyB* and *metD*, so GlyB$^+$ MetD$^+$ transductants were selected by infecting *B. subtilis* IA84 with a transducing lysate prepared

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1   GATATACCTAAATAGAGATAAAATCATCTCAAAAAAATGGGTCTACTAAAATATTATTCCATCTATTACAATAAATTCACAGAATAGTCTTTTAAGTAAG

                                                                  -100
                                          fMet Arg Ser Lys Lys Leu Trp Ile Ser Leu Leu Phe Ala Leu Thr Leu
101 TCTACTCTGAATTTTTTTAAAAGGAGAGGGTAAAGA GTG AGA AGC AAA AAA TTG TGG ATC AGC TTG TTG TTT GCG TTA ACG TTA
                    ----------          ---
    -90                                 -80                                 -70
    Ile Phe Thr Met Ala Phe Ser Asn Met Ser Ala Gln Ala Ala Gly Lys Ser Ser Thr Glu Lys Lys Tyr Ile Val
185 ATC TTT ACG ATG GCG TTC AGC AAC ATG TCT GCG CAG GCT GCC GGA AAA AGC AGT ACA GAA AAG AAA TAC ATT GTC

                        -60                                 -50
    Gly Phe Lys Gln Thr Met Ser Ala Met Ser Ser Ala Lys Lys Lys Asp Val Ile Ser Glu Lys Gly Gly Lys Val
260 GGA TTT AAA CAG ACA ATG AGT GCC ATG AGT TCC GCC AAG AAA AAG GAT GTT ATT TCT GAA AAA GGC GGA AAG GTT
                                                                  ----------
    -40                                 -30                     -20
    Gln Lys Gln Phe Lys Tyr Val Asn Ala Ala Ala Ala Thr Leu Asp Glu Lys Ala Val Lys Glu Leu Lys Lys Asp
335 CAA AAG CAA TTT AAG TAT GTT AAC GCG GCC GCA GCA ACA TTG GAT GAA AAA GCT GTA AAA GAA TTG AAA AAA GAT

                        -10                                 -1   1                                  10
    Pro Ser Val Ala Tyr Val Glu Glu Asp His Ile Ala His Glu Tyr Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln
410 CCG AGC GTT GCA TAT GTG GAA GAA GAT CAT ATT GCA CAT GAA TAT GCG CAA TCT GTT CCT TAT GGC ATT TCT CAA

                                    20                                  30      32
    Ile Lys Ala Pro Ala Leu His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp Ser Gly Ile
485 ATT AAA GCG CCG GCT CTT CAC TCT CAA GGC TAC ACA GGC TCT AAC GTA AAA GTA GCT GTT ATC GAC AGC GGA ATT

                    40                                  50                                  60
    Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp
560 GAC TCT TCT CAT CCT GAC TTA AAC GTC AGA GGC GGA GCA AGC TTC GTA CCT TCT GAA ACA AAC CCA TAC CAG GAC

                64                                  70                                  80
    Gly Ser Ser His Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu Gly Val Ser
635 GGC AGT TCT CAC GGT ACG CAT GTA GCC GGT ACG ATT GCC GCT CTT AAT AAC TCA ATC GGT GTT CTG GGC GTT AGC

                    90                                  100                                 110
    Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly
710 CCA AGC GCA TCA TTA TAT GCA GTA AAA GTG CTT GAT TCA ACA GGA AGC GGC CAA TAT AGC TGG ATT ATT AAC GGC

                                    120                                 130
    Ile Glu Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly Pro Thr Gly Ser Thr Ala Leu
785 ATT GAG TGG GCC ATT TCC AAC AAT ATG GAT GTT ATC AAC ATG AGC CTT GGC GGA CCT ACT GGT TCT ACA GCG CTG

                    140                                 150                                 160
    Lys Thr Val Val Asp Lys Ala Val Ser Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly
860 AAA ACA GTC GTT GAC AAA GCC GTT TCC AGC GGT ATC GTC GTT GCT GCC GCA GCC GGA AAC GAA GGT TCA TCC GGA

                                    170                                 180
    Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala Val Gly Ala Val Asn Ser Ser Asn Gln
935 AGC ACA AGC ACA GTC GGC TAC CCT GCA AAA TAT CCT TCT ACT ATT GCA GTA GGT GCG GTA AAC AGC AGC AAC CAA

                    190                                 200                                 210
    Arg Ala Ser Phe Ser Ser Ala Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr Leu Pro
1010 AGA GCT TCA TTC TCC AGC GCA GGT TCT GAG CTT GAT GTG ATG GCT CCT GGC GTG TCC ATC CAA AGC ACA CTT CCT

                                    220 221                             230
    Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu
1085 GGA GGC ACT TAC GGC GCT TAT AAC GGA ACG TCC ATG GCG ACT CCT CAC GTT GCC GGA GCA GCA GCG TTA ATT CTT

                    240                                 250                                 260
    Ser Lys His Pro Thr Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr Leu Gly Asn Ser
1160 TCT AAG CAC CCG ACT TGG ACA AAC GCG CAA GTC CGT GAT CGT TTA GAA AGC ACT GCA ACA TAT CTT GGA AAC TCT

                                    270
    Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala Ala Ala Gln OC
1235 TTC TAC TAT GGA AAA GGG TTA ATC AAC GTA CAA GCA GCT GCA CAA TAA  TAGTAAAAAGAAGCAGGTTCCTCCATACCTGCTTC
                                                                    ---------------       ---------

1318 TTTTTATTTGTCAGCATCCTGATGTTCCGGCGCATTCTCTTCTTTCTCCGCATGTTGAATCCGTTCCATGATCGACGGATGGCTGCCTCTGAAAATCTTC
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1418 ACAAGCACCGGAGGATCAACCTGCTCAGCCCCGTCACGGCCAAATCCTGAAACGTTTTAACACTGGCTTCTCTGTTCTCTGTC
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FIG. 2. Nucleotide sequence of the subtilisin structural gene and the adjacent regions. The inferred translated amino acid sequence is also shown. The putative ribosome binding sites and transcriptional terminator are the underlined sequences. The *Δapr-684* allele is characterized as a 684-bp deletion mutation beginning with nucleotide 178 and ending with nucleotide 871.

from BG2018. DNA was prepared from several transductants, and introduction of the deletion mutation was confirmed by Southern hybridization experiments (data not shown). When supernatants of *B. subtilis* carrying *Δapr-684* (BG2019) and the wild-type allele (IA84) were assayed for extracellular serine protease activity, BG2019 produced 10-fold less than IA84. This difference was apparent at the 4-, 6-, and 8-h time points in the stationary phase of growth in modified Schaeffer's medium. In addition, strains carrying either the mutant or the wild-type allele were found to produce 80 to 100% heat-resistant endospores after 24 h of growth in modified Schaeffer medium.

TABLE 2. Analysis of the *glyB33*, *catA*, *apr-1*::pIV6, *metD1*, and *argC4* determinants[a]

Phenotype (no. scored)	% of recombinants in the following class[b]											
	A	B	C	D	E	F	G	H	I	J	K	L
Cm^r (87)	47.1	18.4	11.6	11.6		4.6	3.4	1.1	1.1	1.1		
Gly^+ (60)	36.7	13.3	18.3		26.7						3.3	1.1

[a] Strain IA84 (Gly^- Cat^+ Cm^s Met^- Arg^+) was transduced with a PBS1 lysate made from strain BG2021 (Gly^+ Cat^- Cm^r Met^+ Arg^-). See Table 1 for genotypes.

[b] Classes: A, Gly^+ Cat^- Cm^r Met^- Arg^+; B, Gly^+ Cat^- Cm^r Met^+ Arg^-; C, Gly^+ Cat^- Cm^r Met^+Arg^+; D, Gly^- Cat^+ Cm^r Met^+Arg^-; E, Gly^+ Cat^- Cm^s Met^- Arg^+; F, Gly^- Cat^+ Cm^r Met^- Arg^+; G, Gly^- Cat^+ Cm^r Met^- Arg^-; H, Gly^+ Cat^+ Cm^r Met^+ Arg^-; I, Gly^+ Cat^- Cm^r Met^- Arg^-; J, Gly^- Cat^+ Cm^r Met^+ Arg^+; K, Gly^+ Cat^- Cm^s Met^+ Arg^+; L, Gly^+ Cat^+ Cm^s Met^- Arg^+.

DISCUSSION

These data show that the cloned gene is the subtilisin structural gene of *B. subtilis* I168. When this gene was expressed on a high-copy-number plasmid in I168, a fivefold increase in phenylmethylsulfonyl fluoride-sensitive protease activity was observed. The DNA sequence of the gene is 80% homologous to the *B. amyloliquefaciens* subtilisin structural gene (46), and the translated mature coding sequence is 85% homologous to the protein sequence of subtilisin BPN' (21). Furthermore, the subtilisin amino acid active site residues, asp-32, his-64, and ser-221 are conserved in the translated sequence (21). Strongin et al. (42) reported the purification and partial N-terminal amino acid sequence of a *B. subtilis* intracellular serine protease. This protein was only 50% homologous to the N termini of the subtilisins; thus it is unlikely that the cloned gene codes for that intracellular protease. In fact, southern hybridization experiments at low-stringency conditions with the *B. subtilis* subtilisin gene as a probe showed no evidence for a second homologous gene (data not shown).

When an in vitro-derived deletion mutation was introduced into the chromosome of strain BG77, a phenotypic decrease in protease activity was detected on LB plus skim milk agar plates. Supernatants from cultures carrying the subtilisin deletion mutation (BG2019) produced only 10% of wild-type phenylmethylsulfonyl fluoride-sensitive protease levels. A certain level of background protease activity was expected because of cell lysis and release of intracellular serine proteases during the growth of the cultures and because of the secreted esterase (bacillopeptidase F), which has been recently described as a serine protease (33). It was difficult to distinguish between the IA84 wild-type phenotype on LB plus skim milk agar and the phenotype of BG2019, which carries the *Δapr-684* allele. This perhaps is one of the reasons why the isolation of mutations in the structural gene of subtilisin has eluded most investigators to date. However, if the *Δapr-684* allele was introduced into a strain carrying the *prt-77* mutation, the difference between the Apr^- and Apr^+ phenotypes on skim agar was clear. We suspect that the *prt-77* mutation is in the structural gene of the neutral protease, but our analysis is incomplete, and confirmation awaits additional data.

As shown in Fig. 2, assignments were made for a putative Shine-Dalgarno sequence (38) and translational start for the *B. subtilis* I168 subtilisin gene. The necessity for strong complementarity ($\Delta G < -11$ kcal [ca. -46.1 kJ]) between the Shine-Dalgarno sequence of the mRNA and the 3'-OH end of the 16S rRNA for efficient translation in *Bacillus* spp. and *Staphylococcus* spp. genes has been postulated (23, 24, 29). Two possible Shine-Dalgarno sequences were identified within 350 bp of the start of the mature coding sequence and are shown in Fig. 2 as underlined sequences. One sequence (AAAGGCGG) is 129 bp from the start of the mature coding sequence, and the other (AAAGGAGAG) is 330 bp from the start. As calculated by the method of Tinoco et al. (43), the free energies of formation of the most stable double helical pairing between the putative Shine-Dalgarno sequences and the 3'-OH end of rRNA were -11.6 kcal (ca. -48.6 kJ) and -15.4 kcal (ca. -64.5 kJ), respectively. Only the sequence AAAGGAGAG has a translational start codon (GUG) reasonably spaced (9 bp) 3' and in frame with the mature coding sequence. The use of GUG as a translational start codon in procaryotic genes has been reported (11).

It has been proposed (46) that the *B. amyloliquefaciens* subtilisin message is translated as a "prepro" polypeptide. This seems to also be the case for the *B. subtilis* I168 subtilisin. In addition to the 275-amino-acid mature coding sequence, the putative translated polypeptide includes 106 additional amino acids of unknown function. The first 23 amino acids make up a classic signal peptide that includes three positively charged residues within the first 5 amino acids. The following 12 amino acid residues appear to be the hydrophobic core of a signal peptide. A putative signal peptidase site is six residues from the end of the hydrophobic core and follows the tripeptide Ala-Phe-Ser. The signal peptide and signal processing site predictions are based on the consensus data of Perlman and Halvorson (30). The approximately 83 amino acids between the signal sequence and the mature coding sequence is the putative pro sequence; it is unlikely this sequence acts as a signal peptide because 33% of its residues are charged amino acids (69% of these are positively charged), and no reasonable stretch of amino acids resembles a hydrophobic core.

The work of Anderson et al. (3), comparing the nucleotide sequences of human and bovine mitochondrial DNA, provides further evidence that the putative prepro is translated. When homologous translated sequences were examined, they found the least amount of variability in codon position 2, followed by positions 1 and then 3. This relationship deteriorated when untranslated sequences were compared. The *B. amyloliquefaciens* and *B. subtilis* I168 subtilisin sequences were compared for codon position variability; the prepro sequences showed least variability in codon position

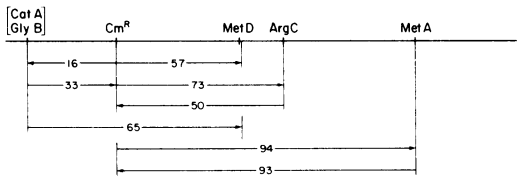

FIG. 3. Linkage relationships among the *catA*, *glyB33*, *apr-1*::pIV6 (Cm^r), *metD1*, *argC4*, and *metA29* markers. Numerical values (100 minus the estimated cotransductional frequencies) are the averages of at least two separate experiments. The arrows point from the selected to the unselected markers.

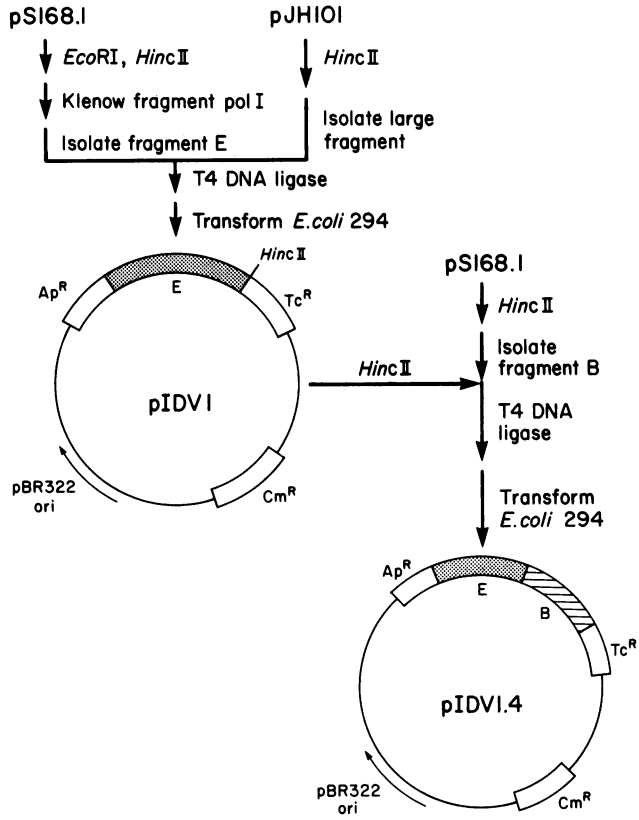

FIG. 4. Construction of the subtilisin deletion plasmid pIDV1.4. Fragments E and B, when ligated in tandem as they appear in Fig. 1, consist of the subtilisin gene with a 684-bp deletion within the translated coding sequence. Plasmid pIDV1.4 contains no origin of replication that functions in *B. subtilis*; when transformed into *B. subtilis*, it integrates via recombination between homologous sequences on the plasmid and the chromosome.

2 (11%), followed by positions 1 (22%) and then 3 (67%). Examination of the mature subtilisin sequences revealed similar numbers (position 2, 12%; position 1, 19%; position 3, 69%).

A promoter sequence for the recognition of RNA polymerase and initiation of transcription could not be identified by homology comparisons with consensus promoter sequences recently described by Johnson et al. (16). However, since the expression of subtilisin is closely associated with the onset of sporulation and many mutations blocking sporulation at early stages affect expression levels of subtilisin (14, 31), transcription of the subtilisin gene is probably under the control of a developmentally regulated promoter. An inverted and repeated sequence ($\Delta G = -22.2$ kcal [ca. 93.0 kJ]; 42) located in the 3′ flanking end of the gene (Fig. 2) may be a transcriptional terminator because similar sequences can be found in termination regions of *E. coli* genes (34) and *Bacillus* spp. genes (37, 46, 47).

The expected pathway for replacement of a chromosomal segment with an altered sequence in *S. cerevisiae* and *E. coli* has been well defined (12, 36, 39). In the case of subtilisin, integration of pIDV1.4 is mediated by a single crossover between fragment E or B and their homologous regions in the chromosome. In either case, both fragments E and B are directly duplicated in the resulting structure. Resolution of the integrated plasmid by a single crossover between one of the two pairs of direct repeats will result either in the restoration of the wild-type gene or a 684-bp deletion within the subtilisin structural gene. Consequently, chloramphenicol-sensitive derivatives of the strain carrying the integrated plasmid must be screened for the mutant phenotype.

An unexpected class of transformants was discovered when competent cells of BG77 were transformed with pIDV1.4. Twenty-five percent of the Cm^r transformants had a protease-deficient phenotype. This class of transformants could not be due to a single crossover integration of pIDV1.4 at either of the homologous regions because the subtilisin gene would not be interrupted. We reasoned that the Cm^r Prt^- phenotype was derived from a double crossover replacement between the homologous chromosomal region and a concatemer of pIDV1.4 (Fig. 5 and 6). This hypothesis seems reasonable since linear plasmid concatemers have been proposed as intermediates in the pathway for plasmid transformation in *B. subtilis* (8). At a minimum, a dimer of pIDV1.4 is required for this replacement pathway. Resolution of this structure by a single crossover between either of the two directly repeated sequences results in the excision of the plasmid DNA and the retention of the deletion within the subtilisin structural gene (Fig. 5 and 6).

In summary, a variation of a technique termed gene replacement was adapted for use in *B. subtilis* I168 to introduce a 684-bp deletion within the subtilisin structural gene. This technique is useful in that defined deletion

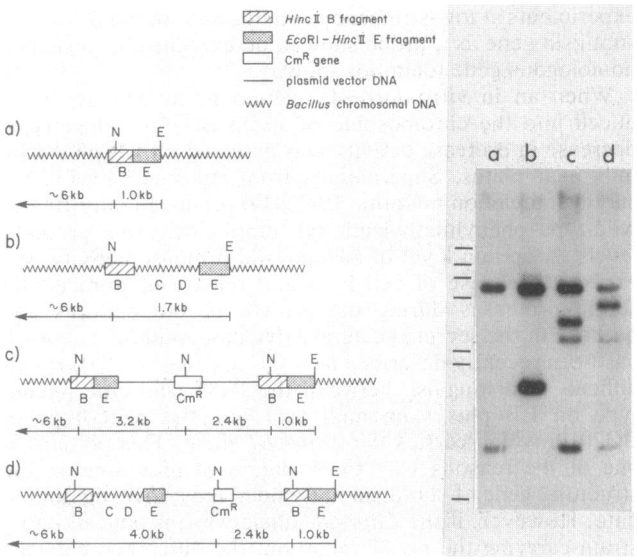

FIG. 5. Structural analysis of plasmid pIDV1.4 integrates and derivatives. Shown are Southern hybridization data and the corresponding proposed structures of wild-type DNA (Cm^s $Prt^{+/-}$, BG77, lane b), DNA from strains carrying the *Δapr-684*::pIDV1.4 allele (Cm^r Prt^-, BG2017, lane c), the *Δapr-684* allele (Cm^s Prt^-, BG2018, lane a), and the *apr*::pIDV1.4 allele (Cm^r $Prt^{+/-}$, BG2016, lane d). The Southern blot shown is composed of *Eco*RI-*Nco*I digests of these DNAs hybridized with the labeled 2.5-kb *Kpn*I-*Eco*RI fragment. The *Eco*RI sites are designated E, and the *Nco*I sites are designated N. The proposed structures show the size and number of bands expected for each of these DNAs. Other digestions and Southern hybridization experiments (data not shown) have confirmed these structures. The horizontal lines next to the Southern blots correspond to fragments 1 through 7 of *Hin*dIII-digested λ DNA, with sizes of 23.1, 9.4, 6.5, 4.3, 2.3, 2.0, and 0.5 kb, respectively.

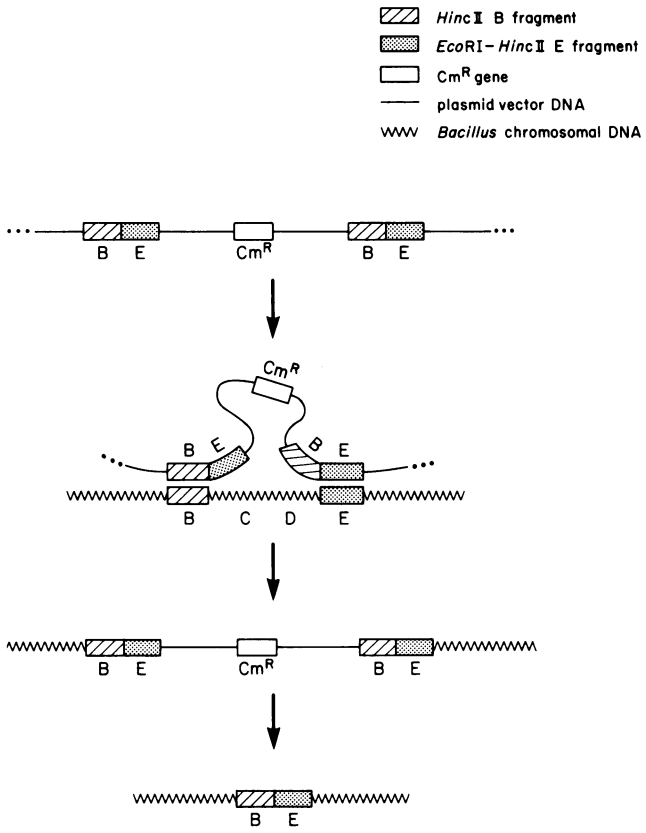

FIG. 6. Possible pathway of deletion formation in *B. subtilis*. A concatemer of plasmid pIDV1.4 interacts via fragments B and E with their homologous regions at the chromosomal subtilisin locus. A double crossover replacement of the wild-type gene with pIDV1.4 and flanking copies of both fragment E and B occurs. As a result of this, fragments C and D are deleted, and fragments E and B are directly duplicated. This structure was manifested phenotypically (Cm^r Prt^-) and confirmed by Southern hybridization experiments (Fig. 5, lane c). Resolution of this structure by a homologous recombination event between the duplicated fragments B or E results in the loss of the plasmid sequence and maintenance of the 684-bp deletion (Fig. 5, lane a).

mutations of cloned genes can be introduced into the chromosome and easily detected by directly screening the phenotypes of plasmid transformants for the desired gene deletion. The plasmid phenotype is unstable because of flanking directly repeated DNA and is lost at a frequency of about 0.1% after about 10 generations of growth in nonselective media, but the deletion mutation is retained. It is apparent from this study that the subtilisin gene product is not required for normal sporulation in *B. subtilis* I168.

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ADDENDUM IN PROOF

Since this paper was submitted, Wong et al. (46a) have reported the DNA sequence of a fragment of the *B. subtilis* subtilisin gene and its map location in the *B. subtilis* chromosome. The results reported by Wong et al. are in agreement with the results we obtained.

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