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Cloning and Expression of the *Bacillus anthracis* Protective Antigen Gene in *Bacillus subtilis*

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The gene encoding the protective antigen (PA) moiety of the tripartite exotoxin of *Bacillus anthracis* was cloned from the recombinant plasmid pSE36 into *Bacillus subtilis* 1S53 by using the plasmid vector pUB110. Two clones, designated PA1 and PA2, were identified which produced PA in liquid cultures at levels of 20.5 to 41.9 μg/ml. This PA was identical to *B. anthracis* Sterne PA with respect to migration on sodium dodecyl sulfate-polyacrylamide gels and to Western blot antigenic reactivity. Addition of lethal factor or edema factor to PA1 and PA2 supernatants generated biologically active anthrax lethal toxin or edema-producing toxin, respectively. The recombinant plasmid in PA1 (pPA101) was 7.8 kilobases, whereas the PA2 strain plasmid (pPA102) was 6.1 kilobases. Both plasmids had deletions extending into the insert sequence but not into the DNA encoding the PA protein. Immunization with the live recombinant strains protected guinea pigs from lethal challenge with virulent *B. anthracis* spores, and the immunization partially or completely protected rats from intravenous challenge with anthrax lethal toxin.

The exotoxin of *Bacillus anthracis* is composed of three polypeptides, protective antigen (PA), edema factor (EF), and lethal factor (LF) (1, 33). The PA component was implicated as being a relevant antigen, i.e., an immunogen providing protection from anthrax infection to those animals immunized with it (2, 8). Unfortunately, even highly purified PA was found to be contaminated with small amounts of EF and LF (19, 21). Indeed, animals immunized with either PA or the licensed human PA vaccine (27) demonstrate titers to the EF and LF components as well as to PA (15, 21). Thus, the protective efficacy of pure PA as well as the influence of the other two components on the immune response to PA remains unclear (15).

In 1983, Vodkin and Leppla reported cloning the PA gene into *Escherichia coli* (37). They ligated into the plasmid vector pBR322 a 6-kilobase (kb) *Bam*HI restriction endonuclease digestion fragment containing the PA gene from the *B. anthracis* Sterne plasmid pXO1. After transformation into *E. coli*, two clones containing the recombinant plasmids, designated pSE24 and pSE36, were identified that produced biologically and serologically active PA, but at a level (5 to 10 ng/ml) 1,000-fold lower than that produced by the Sterne strain. The PA was found in *E. coli* cell lysates but not in culture supernatants. Although the studies verified the location of the PA gene on the pXO1 plasmid, the low levels of PA produced by the clones made study of the *E. coli*-expressed protein difficult. Therefore, research was initiated to move the PA gene into another bacterial species that might produce significantly higher quantities of PA which would be recoverable from the supernatant.

The cloning of the PA gene into *Bacillus subtilis* that is described in this report has been part of the continuing research to develop a more protective and less reactogenic human vaccine against anthrax. The purpose of the study was to produce PA for biochemical studies in large quantities separate from other *B. anthracis* proteins and to generate prototypic live vaccine strains that produce selected *B. anthracis* proteins.

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MATERIALS AND METHODS

Plasmids and bacterial strains. The asporogenic host used as the recipient for transformation with DNA, *B. subtilis* 1S53 (originally designated *B. subtilis* 667 by J. Ito), was obtained from the *Bacillus* Genetic Stock Center, Columbus, Ohio. *B. subtilis* BST1 was the source of the plasmid cloning vector pUB110, which encodes kanamycin resistance. This strain was previously derived (P. Mikesell, B. Ivins, and J. D. Ristroph, unpublished results) by transforming *B. subtilis* 1S53 with pUB110 DNA by the method of Chang and Cohen (3). The recombinant strain *E. coli* HB101 containing pSE36, the source of DNA encoding PA, was the gift of Michael Vodkin (37). *B. anthracis* Sterne was used in the immunization studies. *B. anthracis* Vollum 1B spores were used to challenge guinea pigs in the immunization studies. All bacterial strains were maintained at −70°C in brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) with 12% glycerol.

Isolation and purification of DNA. Plasmid pSE36 DNA was purified by ultracentrifugation in CsCl-ethidium bromide gradients by the methods of Maniatis et al. (23). Sodium dodecyl sulfate (SDS)-NaCl procedures previously described (9, 11, 24) were used to prepare *B. subtilis* plasmid DNA. The DNA preparations were stored frozen at −70°C either in Tris-EDTA buffer (31) or as ethanol-precipitated pellets (23).

Enzymes and synthetic oligonucleotides. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, and calf intestine alkaline phosphatase were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md., New England Bio-Labs, Inc., Beverly, Mass., and Boehringer Mannheim Biochemicals, Indianapolis, Ind., and were used as recommended by the suppliers. Oligonucleotides, 18 nucleotides long, were obtained from Meloy Laboratories, Springfield, Va., and were prepared by the solid phase phosphoramidite method (Applied Biosystems, Foster City, Calif.).

Generation of PA-producing *B. subtilis* clones. Plasmid DNAs were digested with *Bam*HI restriction endonuclease as previously described (23). *Bam*HI-digested pSE36 DNA

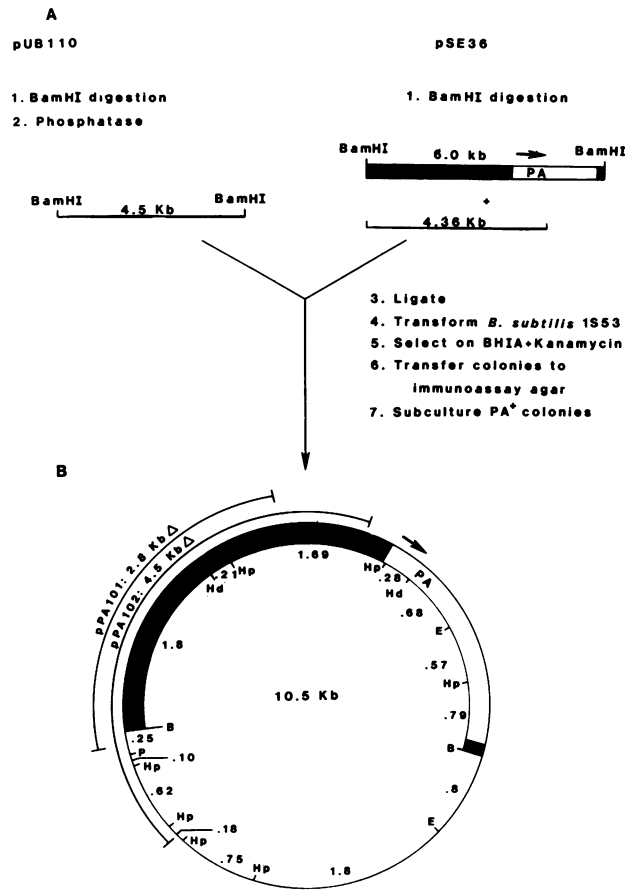

FIG. 1. Cloning scheme and partial restriction map of recombinant plasmids. (A) Vector pUB110 and *E. coli* recombinant pSE36 plasmids were digested with *Bam*HI and ligated. *B. subtilis* 1S53 was transformed with the mixture, and kanamycin-resistant, PA-producing clones were isolated as described in the text. For pSE36: ▬, the 6-kb *Bam*HI fragment, originally cloned from plasmid pXO1 of *B. anthracis* into pBR322; ▬, *E. coli* vector pBR322 DNA (4.36 kb); ▭, portion containing the sequence (2,205 base pairs) encoding PA (unpublished data). The arrow indicates the direction of transcription. (B) Restriction map and deletion analysis of PA-producing recombinants. Plasmids pPA101, pPA102, pUB110, and pSE36 were digested, and the digests were electrophoresed on agarose gels (see Fig. 4). A map of the theoretical 10.5-kb recombinant of pUB110 and the 6-kb PA-producing insert from pSE36 was constructed based on the gel data, on published restriction sites of pUB110 (9, 16) and pSE36 (37), and on the DNA sequence determined for the 4.2-kb *Hin*dIII fragment of pSE36 (unpublished data). Restriction enzymes: B, *Bam*HI; E, *Eco*RI; P, *Pvu*II; Hd, *Hin*dIII; Hp, *Hpa*II.

(60 μg) was ligated to 20 μg of the dephosphorylated pUB110 plasmid digest. Preparation and transformation of competent *B. subtilis* bacteria were performed by the procedures described by Rodriguez and Tait (30), except that 20 μg rather than 1 to 3 μg of ligated DNA was used in the transformation mixtures. The transformation mixture (0.1 ml) was added to plates containing BHI agar with 10 μg of kanamycin per ml and incubated at 37°C for 24 to 72 h. Colonies appearing on the plates were transferred with sterile toothpicks to immunoassay agar (RYEK) plates containing 12 ml of R agar (29), 0.8% yeast extract, 10 μg of kanamycin per ml, and 2 ml of antiserum prepared in goats by immunization with viable spores of *B. anthracis* Sterne. The plates were incubated for 16 h at 37°C in 5% CO_2. Colonies surrounded by precipitin halos were subcultured onto RYEK.

Production and detection of PA in culture supernatants. *B. anthracis* Sterne and *B. subtilis* 1S53 were inoculated onto BHI agar. *B. subtilis* BST1, PA1, and PA2 were inoculated onto BHI agar plus 10 μg of kanamycin per ml. After incubation for 18 h at 37°C, colonies were scraped from the plates and suspended in phosphate-buffered saline (PBS; 6) to a concentration of 10^9 CFU/ml (A_{540}, ~1.4). The suspension (0.1 ml) was inoculated into 125-ml, screw-cap, Erlenmeyer flasks containing 70 ml of either the bicarbonate-containing R medium plus 0.8% yeast extract (RYE) or R medium minus sodium bicarbonate plus 0.8% yeast extract and 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (pH 8.0) (RYEH). Neither medium contained serum. Kanamycin (10 μg/ml) was added to the *B. subtilis* BST1, PA1, and PA2 cultures. The flasks were tightly capped and incubated for 12 h at 37°C with slow shaking (150 reciprocating strokes per min). Cells were removed by centrifugation at 10,000 × *g*, and the supernatants were filter sterilized through cellulose acetate membrane filters (0.22-μ pore size; Millipore Corp., Bedford, Mass.) and frozen at −70°C. Culture supernatants were assayed for PA in microtiter trays by a modification of the double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) described by Vodkin and Leppla (37). The bovine serum albumin components of the blocking and diluent solution were replaced with dried milk (5%) and gelatin (0.5%; Difco), respectively.

SDS-PAGE and Western blot analysis. Culture supernatants were concentrated 10-fold with Centricon-30 microconcentrators (Amicon Corp., Danvers, Mass.). Supernatant proteins were then separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) with 8% acrylamide gels by the method of Laemmli (18). Molecular mass standards in the range of 14,400 to 200,000 daltons were obtained from Bio-Rad Laboratories, Richmond, Calif. Samples were electrophoresed in two gels. One was stained with Coomassie brilliant blue G-250, and the other was electroblotted onto a transfer membrane (GeneScreen; New England Nuclear Corp., Boston, Mass.) as described by Towbin et al. (35). Proteins on the blot that reacted with anti-PA antibody were detected by an ELISA procedure (37) modified as above for the microtiter assay.

Biological activity of supernatant PA. The biological activity of the PA produced by the *B. subtilis* clones in combination with LF or EF was determined. For lethality studies, 20 μg of purified LF (19) was added to each 2-ml aliquot of unconcentrated culture supernatant. The mixtures were injected into the dorsal penile veins of male, 250-g Fischer 344 rats, and times to death (TTD) were noted (7, 13). For edema

TABLE 1. Production of PA in liquid cultures

Strain	PA (μg/ml) in culture supernatant with[a]:	
	RYE	RYEH
B. anthracis Sterne	14.9 ± 0.1	2.3 ± 1.0
B. subtilis 1S53	<1.0	<1.0
B. subtilis BST1[b]	<1.0	<1.0
B. subtilis PA1[b]	20.5 ± 1.6	18.6 ± 4.2
B. subtilis PA2[b]	27.3 ± 6.3	41.9 ± 5.6

[a] Supernatants were assayed for PA by ELISA, and concentrations are given as the arithmetic mean ± the standard deviation.
[b] Cultures contained 10 μg of kanamycin per ml.

studies, 5 μg of purified EF (19) was added to each 0.2-ml aliquot of unconcentrated culture supernatant. Mixtures were injected intradermally into female, 300-g Hartley guinea pigs. After 18 h, the injection sites were examined for redness and edema (20).

Agarose gel electrophoresis. Plasmid DNA from PA-producing *B. subtilis* clones was dissolved in Tris-borate buffer (23) to a concentration of 5 to 15 μg/ml. Plasmid sizes were determined by electrophoresis of the DNA on gels of 0.9% Ultrapure agarose (Bio-Rad) in the Tris-borate buffer. Supercoiled DNA plasmids (Bethesda Research) were used as size standards. Restriction endonuclease digests of plasmid DNA were analyzed by electrophoresis on 0.8 and 1.5% agarose gels (23) in 36 mM Tris base–2.5 mM EDTA–30 mM sodium phosphate (pH 7.8). DNA molecular weight standards were the *Hin*dIII fragments of lambda DNA (Bethesda Research) and the fragments of lambda DNA obtained after double digestion with *Eco*RI and *Hin*dIII restriction endonucleases.

Southern blotting and DNA hybridization. DNA fragments were transferred from agarose gels to Gene Screen Plus hybridization transfer membranes (New England Nuclear) by the technique recommended by the manufacturer. In some experiments, dried gels were prepared for direct hybridization by the method of Tsao et al. (36). ^{32}P-labeled DNA probes were prepared as follows. The lambda DNA *Hin*dIII-*Eco*RI digest fragments were dephosphorylated, and the 5′ ends of these fragments were then labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (New England Nuclear) as described by Maniatis et al. (23). An oligonucleotide complementary to a region of the PA gene was similarly end labeled. Unincorporated ATP was removed by filtration over Sephadex G-50 (Sigma Chemical Co., St. Louis, Mo.) minicolumns (32). Hybridizations were performed essentially as described by Zoller and Smith (40). The labeled oligonucleotide probe and lambda digest DNA fragments (3×10^6 cpm of each) were mixed and added to the prehybridized sheet (membrane or gel) which was then incubated for 48 h at room temperature. The sheet was washed four times with a solution of 0.9 M sodium chloride–0.09 M sodium citrate (pH 7.0) at room temperature for 20 min per wash before autoradiography (40). Nonspecific binding of the labeled lambda DNA fragments to recombinant plasmid DNA or of labeled oligonucleotide to lambda DNA was not observed.

TABLE 2. Biological activity in rats and guinea pigs

Culture supernatant	Rats[a]		Guinea pigs[b]	
	20 μg of LF added	TTD (min)	5 μg of EF added	Edema
B. anthracis Sterne	−	83, 85	−	+
B. subtilis 1S53	+	Survived	+	−
B. subtilis BST1	+	Survived	+	−
B. subtilis PA1	−	Survived	−	−
B. subtilis PA1	+	55, 57	+	+
B. subtilis PA2	−	Survived	−	−
B. subtilis PA2	+	68, 70	+	+

[a] For each group of two rats, RYE culture supernatant (2 ml) with or without LF was injected into rat veins, and the TTD were recorded.

[b] RYE culture supernatant (0.2 ml) with or without EF was injected intradermally into guinea pigs. The injection sites were observed 18 h later for redness and edema.

Immunization with PA-producing *B. subtilis*. Approximately 10^3 CFU of *B. anthracis* Sterne or *B. subtilis* 1S53 was inoculated into 250-ml Erlenmeyer flasks containing 100 ml of BHI. *B. subtilis* BST1, PA1, and PA2 were similarly inoculated into BHI with 10 μg of kanamycin per ml. The flasks were incubated with rapid shaking at 37°C for 12 h.

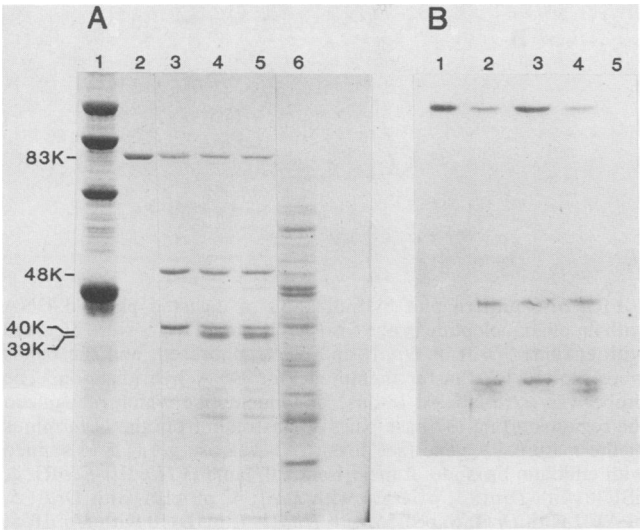

FIG. 2. SDS-PAGE gel and Western blot of *B. subtilis* recombinant strains. (A) RYE broth culture supernatants of recombinant strains were subjected to SDS-PAGE on 8.0% acrylamide gels. Lanes: 1, standard proteins of 116.2, 92.4, 66.2, and 45 kilodaltons; 2, purified PA; 3, PA2; 4, PA1; 5, Sterne strains; 6, BST1. (B) Western blot of PA in supernatants from *B. subtilis* strains. Proteins from another SDS-PAGE gel were transferred and reacted with affinity-purified rabbit anti-PA antibody in an ELISA. Lanes: 1, purified PA; 2, PA2; 3, PA1; 4, Sterne; 5, BST1. The molecular masses of bands reactive with anti-PA antibody are indicated.

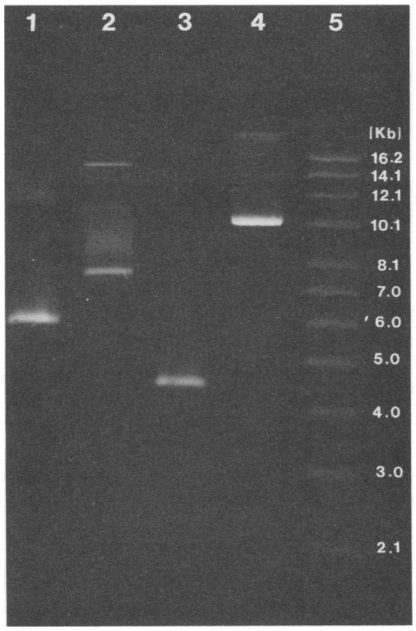

FIG. 3. Purified plasmid DNA. Plasmids pPA101, pPA102, pSE36, and pUB110 were isolated and purified on CsCl-ethidium bromide gradients, electrophoresed on 0.9% agarose gels in Tris-borate buffer, and stained with ethidium bromide. Lanes: 1, pPA102; 2, pPA101; 3, pUB110; 4, pSE36; 5, supercoiled DNA plasmid standards. The fastest migrating band in lanes 1 through 4 was the covalently closed circular plasmid DNA of each sample.

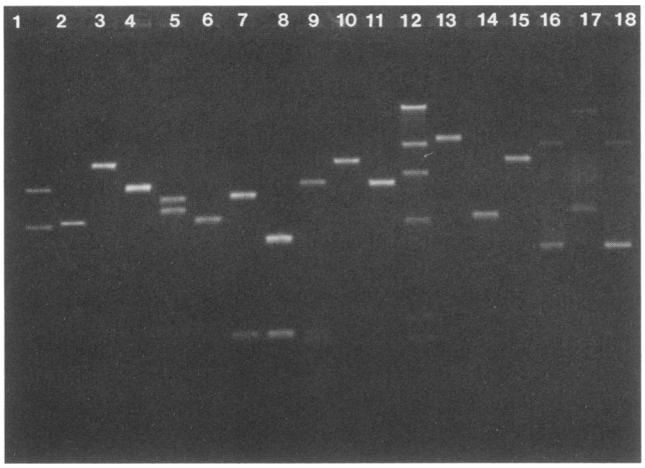

FIG. 4. Restriction digests of plasmid DNA. The digests were electrophoresed on 0.8% agarose gels in Tris-EDTA buffer, and the gels were stained with ethidium bromide. Lanes: 1, pSE36 with *Bam*HI; 2, pUB110 with *Bam*HI; 3, pPA101 with *Bam*HI; 4, pPA102 with *Bam*HI; 5, pSE36 with *Eco*RI; 6, pUB110 with *Eco*RI; 7, pPA101 with *Eco*RI; 8, pPA102 with *Eco*RI; 9, pSE36 with *Hin*dIII; 10, pPA101 with *Hin*dIII; 11, pPA102 with *Hin*dIII; 12, lambda DNA with *Hin*dIII; 13, pSE36 with *Pvu*II; 14, pUB110 with *Pvu*II; 15, pPA101 with *Pvu*II; 16, pPA102 with *Pvu*II; 17, pPA101 undigested; 18, pPA102 undigested. pUB110 was not cleaved by *Hin*dIII (16; data not shown).

The cells were pelleted by centrifugation at 10,000 × *g*, washed once with PBS, and suspended in PBS–12% glycerol. The cell suspensions were frozen in 1-ml aliquots at −70°C. Viable counts (CFU/ml) were determined by making 10-fold dilutions in PBS, plating 0.1 ml in triplicate onto Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.), and incubating the plates for 18 h. Groups of male Fischer 344 rats (250 g) and female Hartley guinea pigs (300 g) were given two intramuscular injections spaced 2 weeks apart of 2×10^6 CFU of one of the strains. After 1 week, 2 ml of blood was withdrawn from each animal by cardiac puncture and the serum was removed and frozen at −70°C. At 2 days after bleeding, the guinea pigs received an intramuscular challenge of 1,500 spores of the virulent Vollum 1B strain. Deaths were recorded, and the harmonic mean TTD was determined (4, 5). At 2 days after bleeding, the rats received an intravenous injection of 50 μg of PA plus 10 μg of LF in 1.0 ml of PBS. TTD were recorded. The prechallenge sera from the immunized animals were examined for antibody to PA in an ELISA (21), and anti-PA titers were determined (15, 38). Statistical analyses of ELISA results were performed as previously described (15, 34).

RESULTS AND DISCUSSION

Clone isolation. The scheme used to clone the *B. anthracis* PA gene into *B. subtilis* is illustrated in Fig. 1A. Approximately 6,000 kanamycin-resistant colonies (3×10^2 transformants per μg of DNA) were identified after transformation of competent *B. subtilis* 1S53 with the ligation mixture. A total of 300 randomly selected colonies were transferred to the RYEK immunoassay agar. After incubation, 10 clones were surrounded by precipitin halos. After five subcultures of these clones on RYEK, two, designated PA1 and PA2, remained stably halo positive. PA1 and PA2 were frozen in BHI with 12% glycerol.

Characterization of PA produced by *B. subtilis* PA1 and PA2. Neither *B. subtilis* 1S53 nor its pUB110-transformed derivative produced detectable PA in either culture medium (Table 1). *B. anthracis* Sterne produced readily detectable PA in the bicarbonate-containing RYE, but the level of supernatant PA produced in the medium lacking bicarbonate (RYEH) was greatly diminished. This finding is in accord with reports from other investigators who detected PA production only in medium containing serum or bicarbonate (8, 14, 20, 26, 28). *B. subtilis* PA1 and PA2 produced readily detectable levels of PA in both RYE and RYEH (Table 1). The production or secretion of PA into the supernatant by PA1 and PA2 was not bicarbonate-dependent, as it was for *B. anthracis*. Two possible reasons for *B. subtilis* PA1 and PA2 apparently producing more PA than *B. anthracis* Sterne in RYE medium (Table 1) are that PA may be more easily secreted by *B. subtilis* than by *B. anthracis* or that there may be more copies of the PA gene in the *B. subtilis* clones than in *B. anthracis* Sterne.

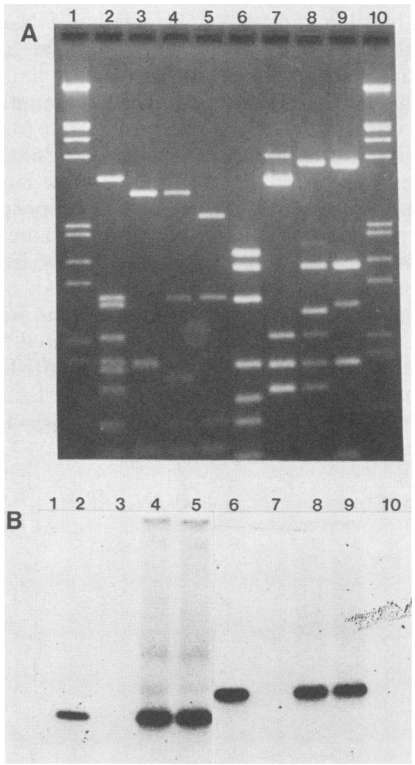

FIG. 5. Southern blot hybridization of digested plasmid DNA with an oligonucleotide probe for PA. Plasmid DNAs were digested with enzymes *Dra*I or *Hpa*II and electrophoresed, and the bands were blotted to transfer membrane for DNA hybridization. The probe was a ^{32}P-labeled 18-base oligonucleotide which recognized the region starting 137 base pairs downstream from the N terminus of the mature PA gene (see text). (A) Agarose gel (1.5%) stained with ethidium bromide. Lanes: 1 and 10, lambda *Hin*dIII-*Eco*RI; 2, pSE36 with *Dra*I; 3, pUB110 with *Dra*I; 4, pPA101 with *Dra*I; 5, pPA102 with *Dra*I; 6, pSE36 with *Hpa*II; 7, pUB110 with *Hpa*II; 8, pPA101 wiht *Hpa*II; 9, pPA102 with *Hpa*II. (B) Autoradiograph of the Southern blot hybridization of the gel in panel A. The oligonucleotide for PA recognized a 1.25-kb band in the *Dra*I digests of pSE36 and the two *B. subtilis* recombinants (lanes 2, 4, and 5) and a 1.54-kb band in the *Hpa*II digests of these three plasmids (lanes 6, 8, and 9). The digest bands of pUB110 and of the lambda *Hin*dIII-*Eco*RI fragments did not hybridize with the probe. Band sizes were determined by hybridization of the gel with ^{32}P-labeled lambda DNA standards. The DNA standard bands in lanes 1 and 10 are not visible in the exposure shown but were visualized upon longer exposure.

After both Coomassie brilliant blue and Western blot staining, the bands from SDS-PAGE gels of PA1 and PA2 supernatants resembled those of the Sterne strain supernatant (Fig. 2). Polypeptide bands reactive with anti-PA antibody had molecular masses of approximately 83,000, 48,000, 40,000, and 39,000 daltons. The 83,000-dalton upper band corresponded with intact PA. Since proteolytic activity was reported in *B. anthracis* (14, 29, 39) and *B. subtilis* (17, 25), the three lower bands may have represented peptide fragments generated by hydrolytic degradation of the intact PA.

B. subtilis PA1 and PA2 supernatants by themselves were nontoxic when injected into Fischer 344 rats (Table 2). When combined with LF, however, they were demonstrably toxic, killing the rats in average times of 56 and 69 min, respectively. Similarly, PA1 and PA2 supernatants mixed with EF produced edema in the skin of guinea pigs, but not when injected alone (Table 2). *B. subtilis* BST1 and 1S53 supernatants had no lethal toxic activity when combined with LF (Table 2) and no edema-producing activity when mixed with EF (Table 2).

Plasmid characterization. Recombinant plasmids pPA101 and pPA102 were isolated from PA1 and PA2, respectively. Ligation of pUB110 with the 6-kb *Bam*HI fragment from *B. anthracis* plasmid pXO1 would have given a predicted 10.5-kb recombinant plasmid. The actual sizes of pPA101 and pPA102 were 7.8 and 6.1 kb, respectively (Fig. 3). These findings are consistent with reports that cloning into *B. subtilis* often results in the loss of recombinant DNA sequences due to deletions (10, 12, 22). Restriction mapping (Fig. 4) verified the extent and location of the deletions in the two plasmids. Both plasmids had large deletions into the insert, which removed one of the *Bam*HI sites joining the insert to vector DNA (Fig. 1B and Fig. 4). The deletion in pPA101 (approximately 2.8 kb) occurred almost exclusively in the pXO1 sequences, whereas the 4.5-kb deletion in pPA102 included about 1 kb of vector DNA. Both recombinant plasmids retained the complete open reading frame for PA (Fig. 1, open bar), including the putative promoter region and signal sequence (S. Welkos, unpublished data). The presence of the intact gene was suggested by hybridization to oligonucleotide probes. In separate hybridization experiments, both plasmid DNAs hybridized to each of three different 18-mer oligonucleotides. One of these recognized the region starting 137 base pairs downstream from the 5′ end of the sequence encoding mature PA. The results of this hybridization are shown in Fig. 5. The second probe was complementary to a region beginning 14 base pairs upstream from the initiation codon for the candidate signal sequence. The third oligonucleotide hybridized to the sequence immediately before the TAA termination codon (data not shown).

Immunization studies. Guinea pigs vaccinated with *B. subtilis* PA1, PA2, or *B. anthracis* Sterne survived challenge with anthrax spores and demonstrated by ELISA significant titers to PA ($P < 0.05$; Table 3). In contrast, guinea pigs which had been immunized with *B. subtilis* BST1 neither survived challenge nor demonstrated significant anti-PA titers. Similarly, only those rats immunized with *B. subtilis* PA1, PA2, or *B. anthracis* Sterne had significant titers to PA ($P < 0.05$) or protection from lethal toxin challenge (Table 4). Rats not immunized or immunized with *B. subtilis* BST1 died in a mean of 47 min, indicating that they received an effective lethal toxin dose of 480 toxic units (13). Rats vaccinated with Sterne or PA1 cells survived the challenge. Rats vaccinated with PA2 cells died after 92 min, and thus received an effective lethal toxin dose of 43 toxic units by the standard curve relating TTD with toxic potency units developed by Haines et al. (13). This extended TTD indicates that over 90% of the lethal toxin injected into this group of rats had been neutralized.

Although these studies appear to suggest that *B. subtilis* PA1 produces less PA in vitro than does PA2 but is a better live vaccine than PA2, recent studies (B. Ivins, unpublished data) indicate that the two strains produce equivalent amounts of PA and are equally effective in eliciting a protective immune response. *B. subtilis* PA1 and PA2 represent prototypes in ongoing efforts to develop improved live vaccines for anthrax. Since PA1 and PA2 do not produce either LF or EF, this study is the first which has conclusively demonstrated that PA produced in the complete absence of EF and LF is protective against challenge by anthrax toxin or spores. Further studies in animals will be done to compare *B. subtilis* PA1 and PA2 with *B. anthracis* Sterne and the human PA vaccine with respect to safety and efficacy. The capability of producing PA in large quantities apart from other *B. anthracis* proteins will facilitate future biochemical studies dealing with such problems as the identification of those sites on the PA molecule responsible for attachment to the cell and for binding EF and LF, delineation of those specific antigenic domains on PA against which protective antibodies can be formed, and elucidation of the anthrax intoxication process at the molecular and cellular levels.

TABLE 3. Immunization of guinea pigs against virulent anthrax spore challenge

Immunization	Survived/challenged	Serologic response[a]
None	0/5	<10
B. subtilis BST1	0/5	<10
B. anthracis Sterne	5/5	29,552[b]
B. subtilis PA1	5/5	6,444[b]
B. subtilis PA2	5/5	1,152[b]

[a] Reciprocal geometric mean anti-PA titers of prechallenge sera as determined by ELISA.
[b] Statistically significant compared to unvaccinated controls ($P < 0.05$).

TABLE 4. Immunization of rats against anthrax toxin challenge

Immunization	TTD (mean)	Serologic response[a]
None	46, 47, 47 (47)[b]	12
B. subtilis BST1	46, 47, 47 (47)[b]	12
B. anthracis Sterne	Survived	48,075[d]
B. subtilis PA1	Survived	14,938[d]
B. subtilis PA2	88, 92, 97 (92)[c]	2,231[d]

[a] Reciprocal geometric mean anti-PA titers of prechallenge sera as determined by ELISA.
[b] Mean TTD indicates rats received an effective dose of 480 toxic units of anthrax toxin (13).
[c] Mean TTD indicates rats received an effective dose of 43 toxic units of anthrax lethal toxin (13). More than 90% of the lethal toxicity was neutralized.
[d] Statistically significant compared to unvaccinated controls ($P < 0.05$).

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