Attenuated Nontoxinogenic and Nonencapsulated Recombinant *Bacillus anthracis* Spore Vaccines Protect against Anthrax

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Several highly attenuated spore-forming nontoxinogenic and nonencapsulated *Bacillus anthracis* vaccines differing in levels of expression of recombinant protective antigen (rPA) were constructed. Biochemical analyses (including electrospray mass spectroscopy and N terminus amino acid sequencing) as well as biological and immunological tests demonstrated that the rPA retains the characteristics of native PA. A single immunization of guinea pigs with 5×10^7 spores of one of these recombinant strains, MASC-10, expressing high levels of rPA ($\geq 100 \mu\text{g/ml}$) from a constitutive heterologous promoter induced high titers of neutralizing anti-PA antibodies. This immune response was long lasting (at least 12 months) and provided protection against a lethal challenge of virulent (Vollum) anthrax spores. The recombinant *B. anthracis* spore vaccine appears to be more efficacious than the vegetative cell vaccine. Furthermore, while results clearly suggest a direct correlation between the level of expression of PA and the potency of the vaccine, they also suggest that some *B. anthracis* spore-associated antigen(s) may contribute in a significant manner to protective immunity.

The etiological agent of anthrax disease in animals and humans is the spore-forming bacterium *Bacillus anthracis*. The major factors of virulence of *B. anthracis* are located on two plasmids, pXO1 and pXO2. pXO2 encodes a poly-D-glutamic acid capsule (19, 41), while pXO1 encodes two binary exotoxins, the lethal toxin (LT) and the edema toxin (ET) (43, 46, 61). These two toxins are composed of three different proteins: protective antigen (PA), edema factor (EF), and lethal factor (LF) (for a review, see reference 36). PA is the common receptor binding domain of the toxins and can interact with the two different effector domains, EF and LF, to mediate their entry into target cells (14). EF is a calmodulin-dependent adenylate cyclase (37) responsible for the edema seen at the site of infection in experimental animals (17). The LF is a metalloprotease (34) recently shown to cleave the amino termini of the mitogen-activated protein kinase kinases 1 and 2, which results in their inactivation (13). It remains to be determined whether these are the main physiological substrates for the LT activity in vivo (5, 22).

Two types of anthrax vaccines are licensed for use in humans: the spores of the toxigenic, nonencapsulated *B. anthracis* STI-1 strain (55) and the cell-free PA-based vaccines consisting of aluminum hydroxide-adsorbed supernatant material from cultures of the toxigenic, nonencapsulated *B. anthracis* strain V770-NPI-R (49) or alum-precipitated culture filtrate from the Sterne strain (6). The use of the live attenuated STI-1 occasionally results in general and local adverse responses, observed both after primary application and revaccination, and the frequency of responses increases with the number of vaccinations (58). Furthermore, it was reported that the STI-1 vaccine has a relatively low immunogenicity (reviewed by Stepanov et al. in reference 58). To increase the immunogenicity, a combined vaccine of live STI-1 supplemented with

cell-free PA formulation was evaluated and proposed for veterinary use (1). While the cell-free PA-based vaccines appear to be safer, they require numerous boosters (8) and were shown to have reduced ability to protect laboratory animals against certain virulent strains of *B. anthracis* (39, 60). In addition, these vaccines contain variable amounts of PA, as well as undefined quantities of LF and EF, adsorbed to aluminum hydroxide (4, 21, 49, 59). It appears, therefore, that there is a need for a safe and more efficient vaccine which could generate stable and prolonged immunity in humans (59). These conclusions led to the evaluation of various adjuvants with purified PA (2, 16, 29, 59) and to the creation of two types of live vaccines: vaccines based on nonvirulent *B. anthracis* (pXO1⁺) mutated strains (31, 47) and vaccines expressing PA from a cloned *pagA* gene using heterologous hosts such as the vaccinia virus, *Bacillus subtilis*, *Salmonella typhimurium* (10, 27, 28, 30, 31, 64), or a nontoxinogenic strain of *B. anthracis* (4). These pioneering studies suggest that recombinant *B. anthracis* live vaccines may have potential as a future anthrax vaccine.

We report here the construction of several recombinant, nonencapsulated, and nontoxinogenic *B. anthracis* spore-forming strains expressing different levels of PA. We demonstrate that one of these strains, containing the *pagA* gene under a potent heterologous constitutive promoter, can be safely used to provide efficacious long-lasting immunity in experimental animals following a single immunization dose.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Bacterial strains used in this study are listed in Table 1. Bacteria were grown (at 37°C, unless stated otherwise) routinely in LB (Luria-Bertani) medium (Difco Laboratories) supplemented with antibiotics (Sigma) as required: ampicillin, 100 $\mu\text{g/ml}$ for *Escherichia coli* cultures; chloramphenicol, 20 $\mu\text{g/ml}$, or kanamycin, 25 $\mu\text{g/ml}$, for bacillus cultures. Preparation of spore stocks was as follows: *B. anthracis* heat-shocked spores were plated and grown overnight, about 40 colonies were pooled and seeded into Schaeffer's sporulation medium broth (53), and the culture was vigorously shaken at 34°C for 72 h. The resulting spores (about 90% of total CFU) were pelleted and washed five times with sterile water. At this stage, microscopic observation revealed only spores. Spores were then heat shocked for 20 min at 70°C and kept at -70°C . Vegetative cells were prepared as described

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TABLE 1. Strains and plasmids used in this study^a

Plasmid or strain	Relevant characteristic(s)	Source and/or reference
Plasmids		
pUB110	Km ^r in bacilli, <i>S. aureus</i> origin of replication	Sigma
pGEM-3Z	Ap ^r in <i>E. coli</i>	Promega
pPA20-N	<i>pagA</i> promoter region fused to <i>pagA</i> gene and inserted in pGEM-3Z, Ap ^r	This work
pPA20-α	α-Amylase promoter fused to <i>pagA</i> gene and inserted in pGEM-3Z, Ap ^r	This work
pRIT5	Ap ^r in <i>E. coli</i> , Cm ^r in gram-positive organisms, pC194 origin of replication	Pharmacia (44)
pASC-1	<i>pagA</i> promoter and coding regions of pPA20-N fused with pRIT5 backbone, Ap ^r Cm ^r	This work
pASC-α	α-Amylase promoter and <i>pagA</i> gene of pPA20-α fused with pRIT5 backbone, Ap ^r Cm ^r	This work
pAUB	pPA20-N fused with pUB110, Ap ^r in <i>E. coli</i> , Km ^r in <i>E. coli</i> and bacilli	This work
pAUB- <i>atxA</i>	<i>atxA</i> gene cloned in pAUB, Ap ^r Km ^r	This work
pAαUB	pPA20-α fused with pUB110, Ap ^r in <i>E. coli</i> , Km ^r in <i>E. coli</i> and bacilli	This work
pAαUB-SD8	pAαUB with the wt <i>pagA</i> SD sequence AAGGAGA, Ap ^r Km ^r	This work
pAαUB-SD10	pAαUB with <i>B. subtilis</i> consensus SD sequence AAGGAGG, Ap ^r Km ^r	This work
Strains		
<i>B. anthracis</i>		
Vollum	PA ⁺ LF ⁺ EF ⁺ (pXO1 ⁺) Cap ⁺ (pXO2 ⁺)	ATCC 14578
V770-NP1-R	PA ⁺ LF ⁺ EF ⁺ (pXO1 ⁺) Cap ⁻ (pXO2 ⁻) nonproteolytic	ATCC 14185
Δ14185	PA ⁻ LF ⁻ EF ⁻ (pXO1 ⁻) Cap ⁻ (pXO2 ⁻) derivative of ATCC 14185	This work (19, 52)
MASC-10	Δ14185 electroporated with pAαUB, PA ⁺ Km ^r	This work
MASC-10-08	Δ14185 electroporated with pAαUB-SD8, PA ⁺ Km ^r	This work
MASC-10-10	Δ14185 electroporated with pAαUB-SD10, PA ⁺ Km ^r	This work
MASC-20	Δ14185 electroporated with pAUB, PA ⁺ Km ^r	This work
MASC-40	Δ14185 electroporated with pAUB- <i>atxA</i> , PA ⁺ AtxA ⁺ Km ^r	This work
<i>B. subtilis</i>		
DB104	<i>nprE18 ΔaprA3</i>	33
WB600	<i>ΔnprE ΔaprE Δepr Δbpf Δmpr ΔnprB</i>	67
<i>E. coli</i>		
HB101	<i>recA13</i>	Promega
GM2929	<i>dam13::Tn9 (Cm^r) dcm-6</i>	NEB

^a Abbreviations: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Cap, anthrax capsule proteins; ATCC, American Type Culture Collection; NEB, New England Biolabs; wt, wild type.

(31) with some modifications. Several colonies from overnight growth were pooled and seeded into LB medium with kanamycin, and the culture was allowed to grow up to an A_{550} of 4. Microscopic observation revealed only vegetative cells.

Recombinant DNA and general techniques. Standard procedures were used throughout the study (23, 42). Enzymes were purchased from New England Biolabs or Promega and were used as recommended by the supplier. For PCR amplifications, Vent or *Tli* DNA polymerases were used. *Bacillus* cells were prepared and electrotransformed as described (9). Plasmid DNA for electroporation of *B. anthracis* Δ14185 and *B. subtilis* was isolated from *E. coli* GM2929 (51) and HB101, respectively. Plasmid DNA was extracted from *E. coli* with Wizard Plus Maxiprep or SV Miniprep kits (Promega). *B. subtilis* and *B. anthracis* plasmid DNA was extracted in a similar fashion, except that prior to lysis, cells were prewashed (50 mM Tris-HCl buffer, pH 7.4, 10 mM EDTA) and were then incubated (at 37°C) for 30 min in 7 mg of lysozyme (Sigma) per ml and 0.2 M sucrose (Merck). DNA sequences were determined with the ABI rhodamine termination reaction kit (ABI310 Genetic Analyzer; Applied Biosystems). Oligonucleotides were constructed by using the Applied Biosystems 392 DNA/RNA synthesizer.

Construction of plasmids. Plasmids and their relevant characteristics are listed in Table 1 and Fig. 1. The coding region of *pagA* was PCR amplified from Sterne DNA template using oligonucleotides 5'-GTATATGAAAAACGAAAGTG TTAATACC-3', carrying the sequences of the translational start site of *pagA* (nucleotides 1800 to 1829 by the numbering of Welkos et al. [65]), and 5'-GG ATCTACAACAATCTCAAAGG-3' (complementary to nucleotides 4211 to 4235, which are located downstream from the transcriptional terminator of *pagA* [25, 65]).

The *pagA* promoter region was PCR amplified by using oligonucleotides 5'-GGATCCATGTTTCAAGGTACAATAATTATG-3' (positions 1412 to 1436, downstream from open reading frame 1 and from the long palindromic sequence [65]) and 5'-TCTAGATACGTACTCTTTTGTATAAA-3' (positions 1780 to 1803, carrying a T-to-A substitution [position 1797] to generate a *Sna*BI site). The PCR products of the *pagA* promoter and coding regions were digested with

*Bam*HI-*Sna*BI and *Bam*HI, respectively, and were ligated into *Bam*HI-digested pGEM-3Z to generate pPA20-N.

To construct pPA20-α, the *pagA* promoter region from pPA20-N was exchanged with a *Sali*-*Sna*BI 163-bp synthetic DNA fragment which carries the promoter region of the α-amylase gene of *Bacillus amyloliquefaciens* (positions 17 to 151 [45]) and the Shine-Dalgarno (SD) signal of *pagA*.

The *Staphylococcus aureus*-derived plasmids (i.e., pUB110 and pRIT5, a derivative of pC194 [Table 1]), described previously as expression vehicles for PA in *B. anthracis* and *B. subtilis* (30, 51, 56), were used in this study. Specifically, plasmids pASC-1 and pASC-α were generated by using *Aat*II-*Bam*HI sites for cloning the *pagA* gene either from pPA20-N or pPA20-α into pRIT5. Plasmids pAUB and pAαUB were obtained by inserting pUB110 into the *Bam*HI site of pPA20-N or pPA20-α, respectively (Fig. 1). To construct pAUB-*atxA*, the entire *atxA* gene and flanking regions were PCR amplified (positions 616 to 2609 [62]) and were cloned into the *Aat*II site clockwise and upstream to *pagA*. The SD-derivative plasmids, pAαUB-SD8 and pAαUB-SD10, were constructed by replacement of the SD region in pAαUB with a synthetic DNA fragment carrying the modified SD signals (Table 1). All DNA constructs were isolated from at least two independent clones and were verified by sequence analysis.

Plasmid stability. To determine the stability of plasmids derived from pUB110 in the vegetative Δ14185 host, cells were initially grown in selective medium (5 μg of kanamycin per ml) to mid-log phase (A_{550} = 0.3). Cells were diluted 1:100 without antibiotics into RMM medium and allowed to grow to the end of the log phase. After five to seven doubling times, cells were diluted again in Schaeffer's sporulation medium without antibiotics. Altogether, vegetative cells were propagated for 10 to 12 generations without antibiotics before entering the sporulation stage. After 3 days in culture, spores were collected, heat shocked, and plated on LB agar with or without kanamycin, and colonies were counted (approximately 100 to 200).

Production, purification, and formulation of rPA. For production of recombinant PA (rPA), *B. subtilis* and *B. anthracis* cultures were grown in modified FA medium (3.3% tryptone, 2% yeast extract, 0.74% NaCl, 0.4% KH₂PO₄, 0.8% Na₂HPO₄, 2% glycerol, pH 8 [57]) up to an A_{550} of 8 to 11 under vigorous

FIG. 1. Cloning scheme of PA-expression vectors. pGEM-3Z *E. coli* vector carrying the *pag* gene (PA) under either the *Ppag* promoter (pPA20-N) or the *Pa-amy* (*Pa-amy*) promoter (pPA20-α) was used to construct four shuttle vectors for expression of PA in *Bacillus*. The vectors pAUB and pAαUB were based on a pUB110 plasmid, and the vectors pASC-1 and pASC-α were based on pRIT5. These plasmids carry different origins of replications (ori) of plasmids isolated from gram-positive bacteria and from *E. coli*. mcs, multiple cloning site; Amp, ampicillin resistance in *E. coli*; Km and Cm, kanamycin and chloramphenicol resistance genes, respectively, in *Bacillus*. Only relevant restriction sites for construction are presented. For details, see text.

agitation (aerobic conditions). Semianaerobic growth conditions were achieved by growing *B. anthracis* in 250-ml screw-cap Erlenmeyer flasks (Corning) containing 150 ml of 0.4% bicarbonate-containing RMM medium (38) (5 µg of kanamycin per ml was added for recombinant strains). The flasks were tightly capped and incubated for about 12 h at 34°C with slow shaking. Phenylmethylsulfonyl fluoride, EDTA, and 1,10-phenanthroline (all purchased from Sigma) were added to final concentrations of 2, 2, and 0.5 mM, respectively, upon harvesting. The supernatant was collected by centrifugation and was filtrated through cellulose acetate filter systems (0.2-µm pore-size filters; Corning) for large volumes, or Acrodisc filters (0.2-µm pore-size filters; Gelman Sciences) for small volumes. Samples were frozen and stored at -70°C.

Purification of rPA or native PA was carried out on a Mono-Q column (50). For preparation of a cell-free vaccine, purified PA was adsorbed to Alhydrogel (Superfos Biosector a/s), as previously described (29), to a final concentration of about 50 µg per ml.

SDS-PAGE and immunoblot analysis. PA highly specific polyclonal antiserum (anti-PA-βgal) was prepared by using a polypeptide chimera of PA (amino acids 453 to 512) and β-galactosidase as an antigen. The cloning of PA-βgal into the *E. coli* expression vector pTOZ and the subsequent purification of the protein and immunization of mice were performed as described (20). High-titer (>1:100,000) anti-PA antiserum was prepared by using purified PA formulated with alum. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were carried out as described previously (54), except that for immunoblotting, Hybond-C pure membrane (Amersham Life Science) and horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (Sigma) were used for the development of blots.

Determination of the molecular mass of PA by LC ESI MS. Molecular mass measurements were carried out on a VG Platform liquid chromatography (LC) mass spectroscopy (MS) instrument, which consists essentially of a high-pressure liquid chromatograph online with an electrospray ion (ESI) source operating at atmospheric pressure, followed by a quadrupole mass analyzer. The analysis was performed by injecting PA preparations into an R1/H perfusion column (2.1 by 30 mm; Poros, Ltd.) by using a CH₃CN-trifluoroacetic acid (0.1%) gradient, at a flow rate of 0.2 ml/min. The flow rate to the ESI source was set to 0.02 ml/min.

N-terminal amino acid sequence analysis. N-terminal sequence analyses of PA samples were performed by the Gas Phase Protein Sequencer (Model 477A;

Applied Biosystems). Phenylthiohydantoin-amino acids were analyzed offline by reversed-phase high-pressure liquid chromatography (C₁₈) (24).

Biological activity of rPA. The rPA was tested for cytotoxicity in the macrophage lysis assay with J774A.1 murine cells as described previously (18, 40). The rPA preparations were subjected to twofold dilutions in the presence of purified LF (2 µg/ml). From each dilution, 10 µl was added to a 96-well tissue culture plate containing 5 × 10⁴ J774A.1 cells/well in 100 µl of Dulbecco's modified Eagle medium supplemented with 5% fetal calf serum (Biological Industries, Beit Haemek, Israel). Plates were incubated for 3 h at 37°C in 7.5% CO₂. Following the addition of 10 µl of 0.75% MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) solution in phosphate-buffered saline (PBS) to each well, plates were incubated for an additional 1 h under the same conditions. Optical absorbance (540 nm) was determined after treatment (30 min) with acidified (0.8% HCl) isopropanol.

The *in vivo* biological activity of LT and ET composed of rPA with purified LF or EF, respectively, was determined and compared to that of identical toxins prepared with native PA. Lethality experiments in Fischer 344 rats (body weight, 200 to 300 g) were performed as described previously (56) with mixtures of PA (40 µg) and LF (8 µg). For ET toxicity, 0.5 µg of purified EF was added to 0.5 µg of PA and injected intradermally into female guinea pigs, and the injection sites were examined (after 18 h) for redness and edema as described previously (38).

Experimental animals and evaluation of virulence and attenuation of *B. anthracis* strains. All animals used in this work were obtained from Charles River Laboratories (Sulzfeld, Germany) and were cared for according to the 1997 guidelines of the National Institutes of Health for the care and use of laboratory animals, and the experimental protocols were approved by the Animal Use Committee of the Israel Institute for Biological Research. For evaluation of virulence or degree of attenuation, female Hartley guinea pigs, weighing 220 to 250 g, or ICR mice (25 g) were injected subcutaneously (s.c.) with the indicated dose of spores and were observed for 3 weeks after injection.

Immunization and challenge of guinea pigs. Female Hartley guinea pigs, weighing 220 to 250 g, were immunized s.c. with a single dose of spores, or vegetative cells, of *B. anthracis* strains. At indicated time intervals following the single immunization, eight animals were bled by cardiac puncture for serological studies and eight animals were challenged with 20 to 30 50% lethal doses (LD₅₀s)

TABLE 2. Modulation of PA production in *B. subtilis* and *B. anthracis* strains by gene dosage, transcription, and translation signals

Host and strain ^a	Expression vector ^b			PA yield ^c (μ g/ml)
	Plasmid name ^a	Promoter origin	Replication origin	
<i>B. subtilis</i>				
DB104	pASC-1	<i>pag</i>	pC194	2
	pASC- α	α - <i>amy</i>	pC194	6
	pA α UB	α - <i>amy</i>	pUB110	35
WB600	pAUB	<i>pag</i>	pUB110	33
	pA α UB	α - <i>amy</i>	pUB110	100
<i>B. anthracis</i>				
MASC-20	pAUB	<i>pag</i>	pUB110	7
MASC-10	pA α UB	α - <i>amy</i>	pUB110	115
MASC-10-08	pA α UB-SD8	α - <i>amy</i>	pUB110	120
MASC-10-10	pA α UB-SD10	α - <i>amy</i>	pUB110	110

^a For strain and plasmid descriptions see Table 1. *B. subtilis* DB104 (kindly provided by R. Doi) and WB600 (kindly provided by S.-L. Wong) strains are deficient in two and six extracellular proteases, respectively.

^b pUB110 and pC194 replicate in *B. subtilis* in about 50 and 15 copies/chromosome, respectively (9). Abbreviations: *pag*, *pagA* promoter region; α -*amy*, α -amylase promoter.

^c The levels of PA were determined by the cytotoxicity assay (see Materials and Methods). Quantitation by capture ELISA using anti-PA antibodies raised against highly purified PA generated similar values (data not shown).

of *B. anthracis* Vollum (LD₅₀ = 100 spores; prior to challenge the spores were heat shocked for 20 min at 70°C). Animals were observed for 2 weeks after challenge. Statistical analysis was performed by using the comparison binomial proportion test.

For immunization with recombinant or native PA, the guinea pigs were injected s.c. with 0.5 ml of the cell-free alum-adsorbed PA (see Materials and Methods) at days 0, 14, and 28. Two weeks after the final immunization, animals were challenged as described above or were used to determine anti-PA antibody titers.

Serological tests. Enzyme-linked immunosorbent assays (ELISAs) for detection of anti-PA, anti-core, and anti-exosporium antibodies were carried out by coating 96-well microtiter plates (Nunc) with the relevant antigens: for anti-PA antibody, 350 ng of rPA (obtained from culture supernatant of rPA-producing *B. subtilis* WB600 cells) per well; for anti-core antibody, 180 ng of Δ 14185 core antigens (representing the crude soluble material of vegetative Δ 14185 cells extracted as described [15]) per well; and for anti-exosporium antibody, 50 ng of exosporium antigens prepared essentially as described previously (7) per well. Rabbit anti-guinea pig immunoglobulin G conjugated to alkaline phosphatase (Sigma) was used in all ELISAs, and titers (from twofold dilutions) were determined as the highest serum dilution displaying a value twofold over background by using a Thermomax microplate reader (405 nm). Neutralizing antibodies were determined by virtue of their ability to prevent death of J774A.1 cells by LT as described (40). Antibody titers were calculated as reciprocal geometric mean titers (GMT).

Nucleotide sequence accession number. The sequence for the *pagA* gene appears in GenBank under accession no. AF268967.

RESULTS

Modulation of *pagA* gene expression in various bacillus strains. The *pagA* coding sequence was derived from *B. anthracis* Sterne and was cloned as described in Materials and Methods. To modulate the level of production of the rPA, the coding sequence of the *pagA* gene was cloned under two regulatory elements: the native *pagA* promoter region and the α -amylase promoter from *Bacillus amyloliquefaciens*. The *pagA* regulatory sequences were PCR cloned from the Sterne strain and span the proposed AtxA binding site and the two major and minor transcription start sites, P1 and P2, respectively (12, 35). The α -amylase promoter was synthetically generated based on the published sequence and contains the preceding inverted repeat structure suggested to prevent transcription from signals upstream of the α -amylase promoter (32, 45). In addition, the SD signal was modified in some of the constructs (Table 1).

The different *pagA*-containing constructs described above were introduced into two prototype *E. coli*-*Bacillus* shuttle vectors which differ in their copy numbers (Tables 1 and 2 and Fig. 1). Each of these plasmids was used to transform two *B. subtilis* strains, DB104 and WB600, differing in their extracellular protease contents. In addition, a pXO1⁺ pXO2⁺ *B. anthracis* strain (Δ 14185) derived from the low-proteolytic V770-NP1-R strain (66) was used as a host for rPA expression. The levels of rPA secreted into the medium by the various strains were determined by ELISA as well as by the cytotoxicity assay. The latter, unlike the ELISA, provides information which is not obscured by degradation products of PA. The results, summarized in Table 2, demonstrate that rPA can be expressed efficiently in all the bacillus strains tested. High levels of rPA expression (over 100 μ g/ml) driven by the α -amylase promoter are attained in both *B. subtilis* and *B. anthracis* strains. The comparison of the efficiency of the two promoters in *B. subtilis* background, using vectors with the same copy number, suggests that the α -amylase promoter generates at least threefold-higher yields of rPA than the *pagA* promoter (Table 2). In the *B. anthracis* Δ 14185 strain, carrying the PA gene under the constitutive α -amylase promoter (MASC-10), the levels of the secreted rPA were at least 10-fold higher than those expressed by the native promoter (MASC-20).

The different behavior of the native *pagA* promoter in the two bacterial backgrounds (*B. subtilis* and *B. anthracis*) may be due to the tight control of *pagA* expression in *B. anthracis* (25, 26, 35). The low level of rPA expression driven by the *pagA* promoter region in strain MASC-20 is most likely due to the lack of positive, as well as negative, regulatory signals in the *B. anthracis* Δ 14185 host. Transcription of the *pagA* gene is known to be initiated from either P1 or P2 (35). However, CO₂-bicarbonate growth conditions induce transcription exclusively from P1, which is dependent upon the *trans*-acting positive regulator, AtxA, encoded by the *atxA* gene (62). To exploit some of these natural signals for modulation of PA expression, we cloned the positive-regulator AtxA gene and introduced it on the same plasmid containing the *pagA* coding sequence with the native *pagA* promoter region (MASC-40) (Table 1). The effect of AtxA on PA production was evaluated under semi-

FIG. 2. Effect of AtxA on rPA expression under different growth conditions. (A) Western blot analysis (using anti-PA antiserum) of culture supernatants from *Ppag* *Atx*⁺ (MASC-40) and *Ppag* *Atx*⁻ (MASC-20) strains. Cells were grown under semianaerobic conditions to an A_{550} of 1, and equivalent amounts of culture supernatants were applied on SDS-PAGE gels. (B) Coomassie blue-stained SDS-PAGE of samples from culture supernatants of MASC-40, MASC-20, and *Pα-amy*ase *Atx*⁻ (MASC-10) strains grown to an A_{550} of 11 under aerobic conditions. The concentration of PA in each culture was determined by the cytotoxicity assay. The bands corresponding to PA are indicated by an arrow. Abbreviations: *Ppag*, *pagA* promoter region; *Pα-amy*, *α-amy* promoter.

anaerobic or aerobic conditions (Fig. 2). As expected, under semianaerobic conditions, AtxA enhances production of rPA about 20-fold (Fig. 2A). Under aerobic conditions (Fig. 2B), AtxA still allowed some enhancement of rPA production (threefold). Dai and Koehler (11) found that high levels of AtxA expression from high-copy-number plasmids actually reduced expression of PA, but this was manifested in a pXO1 background. Under any of the conditions tested, levels of expression of rPA from MASC-20 or MASC-40 were lower than those obtained by MASC-10, where expression is driven constitutively from the *α-amy* promoter.

B. subtilis requires a stringent SD signal for efficient translation initiation (3), but to the best of our knowledge a prototype consensus SD signal for *B. anthracis* was not described. The efficiency of rPA expression in both *B. subtilis* and *B. anthracis* was determined by comparing the *B. subtilis* consensus SD sequence (AAGGAGG [63]) to the putative native *pagA* SD (AAGGAGA [65]) and a derivative thereof (AAGGAGT) (Table 1). No significant differences in expression levels of rPA were observed among *B. anthracis* strains carrying these three different SD signals (Table 2). Finally, as reported previously (2, 16, 56), bacterial proteases secreted into the medium can affect the yields of rPA (Table 2). Comparison of PA production from *B. subtilis* WB600 and DB104 demonstrates that higher yields of PA are obtained from WB600, a six-protease-deficient strain (Table 2). In addition, we note that SDS-PAGE analyses of supernatants from cultures of the different protease-deficient strains of *B. subtilis* (DB104 and WB600) and the low-proteolytic *B. anthracis* ATCC 14185 strain exhibit different PA degradation patterns (Fig. 3), probably reflecting the differential protease content in these cultures.

To estimate the extent of stability of the pUB110-derived plasmids (Table 1) in the *B. anthracis* Δ14185 strain, we propagated the vegetative cells in nonselective medium for 10 to 12 generations. Since the vegetative cells appear as a multicell chain (which can introduce a bias to apparently higher values of plasmid stability), we allowed the cells to sporulate, and thus were able to score plasmid stability in individual cells (see

FIG. 3. Production of rPA from protease-deficient strains of *B. subtilis* and *B. anthracis*. Western blot analysis (using anti-PA-β-gal antiserum) of culture supernatants obtained from *B. subtilis* DB104 and WB600 strains carrying pAαUB and from *B. anthracis* ATCC 14185 (pXO1⁺) (lanes 1, 4, and 7, respectively) or dilutions of 1:10 (lanes 2, 5, and 8), 1:50 (lanes 3 and 6), and 1:25 (lane 9). The bands corresponding to PA are indicated by an arrow. PA levels (as determined by the cytotoxicity assay) in the supernatants of the pAαUB-transformed DB104 and WB600 strains (aerobic cultures) and the ATCC 14185 strain (semianaerobic culture) were 35, 90, and 20 µg/ml, respectively.

Materials and Methods). More than 80% of the colonies grown from spores on nonselective plates contained the kanamycin resistance marker carried on the plasmid. From these plates, 10 randomly selected colonies were found to retain the ability to produce PA at levels similar to those obtained from cells which remained under selection throughout the entire procedure. These results, together with the restriction enzyme profile analysis (not shown), suggest that the entire plasmid is maintained in intact form. The observed relatively high stability of the pUB110-derived plasmid carrying the *pagA* gene in our constructs may be due to the unique orientation of the *pagA* gene or due to the fact that our constructs lack the previously reported sequence upstream of *pagA* which was suggested to introduce genetic instability (2).

Biochemical and biological characterization of the rPA. DNA sequencing of the cloned *pagA* gene (derived from Sterne), which was used to generate the various MASC vaccine strains, shows a single nucleotide change (G to C at position 2743) from the published sequence (65). This change (resulting in a codon change from Glu to Gln at amino acid position 285 in the mature polypeptide) was also found by direct PCR amplification and sequencing of the gene from the Sterne strain. This difference in nucleotide sequence of *pagA* was also reported recently by others (2). We have also sequenced the *pagA* gene from ATCC 14185 and found that it belongs to the PA genotype V (48). This ATCC 14185 *pagA* gene carries two nucleotide changes in sequence compared to the Sterne *pagA* gene, one of which results in an amino acid change (Ala to Val; position 599 in the mature polypeptide).

The PA proteins produced from either MASC-10 or ATCC 14185 cells were purified by chromatographic procedures and

TABLE 3. Molecular characterization of rPA

PA protein ^a	Molecular mass (Da)			N-terminal sequence
	Mass calculated ^b	Mass ESI MS	ΔMass	
Native	82,701	82,720	+19	EVKQENRLLNE
Recombinant	82,673	82,675	+2	EVKQENRLLNE
Recombinant ^c	82,673	82,075	-598	NRLLE

^a Native and recombinant PA were purified (see Materials and Methods) from ATCC 14185 and MASC-10 cultures, respectively. The recombinant PA originating from the Sterne *pagA* gene differs in sequence from the native PA by one amino acid at position 599 (see text).

^b The expected MW was calculated by using the PeptideSort program of the Wisconsin Package Version 9.0 (Genetics Computer Group, Madison, Wis.) according to the ATCC 14185 and Sterne *pagA* DNA sequences determined in the present study.

^c Recombinant PA was purified without the addition of protease inhibitors.

were subjected to different analyses. Mobility of the rPA product on SDS-PAGE was indistinguishable from that of native PA (Fig. 3). A molecular mass of 82,675 Da was determined by ESI MS for rPA which is in excellent agreement with the calculated molecular weight (MW) (Table 3). The measured MW of the native PA (produced by the ATCC 14185 bacteria) was higher than that measured for rPA as could be expected from the differences in their DNA sequences. In addition, we observed that while the N-terminal amino acid sequences of rPA and native PA were identical (Table 3), the latter was less susceptible to Edman cleavage. This observation suggests that the amino terminus of the native PA molecule contains some modification (e.g., formylation) which makes it more resistant to degradation and thus could account for the small difference in MW (+19) between the calculated and measured mass of the native PA molecule. Another observation worth noting is that rPA collected from the supernatant of stationary MASC-10 bacterial cultures had a molecular mass lower by 598 Da than expected. The N-terminal sequence (Table 3) revealed that the first five amino acids of mature PA are truncated under such conditions and that the cleavage of these five amino acids can fully account for the observed mass decrease.

This mass loss could be prevented by the addition of protease inhibitors during the purification process.

The biological functions of the rPA were compared to those of native PA by replacing the latter in LT and ET assays. A mixture of rPA (purified from a culture of MASC-10) with LF lysed 50% of macrophage-like J774A.1 cells at the same concentration as native PA. Interestingly, the N terminus-truncated rPA version (see above) was as potent as intact rPA or native PA in this biological assay. These results suggest that the five terminal amino acid residues are not essential for PA function in the LT cytotoxic assay. Exposure of Fisher 344 rats to LT composed of either rPA or native PA resulted in a similar time to death, which is in good agreement with previous reports (56). In addition, similar concentrations of rPA or native PA were needed to exhibit edema in the skin of guinea pigs when injected intradermally with EF (see Materials and Methods).

In view of the similarities between native PA and rPA in all the tests described above, it was expected that both types of PA preparations would be indistinguishable in their immunogenicity. Indeed, following a three-dose immunization schedule (see Materials and Methods) of alum-formulated vaccines of rPA and native PA, both vaccines elicited in guinea pigs similar anti-PA antibody titers in ELISA (1:50,000 to 1:250,000) or in neutralization assays (1:20,000 to 1:70,000). Both rPA and native PA vaccines conferred full protection against a 20-LD₅₀ challenge of the Vollum strain.

Evaluation of recombinant bacilli as live attenuated vaccines. The available live attenuated anthrax vaccines, such as STI-1 and Sterne, are relatively virulent (59). For example, inoculation of guinea pigs with one dose of 10⁷ Sterne spores or a single dose of 5 × 10⁵ STI-1 spores was reported to result in the mortality of 30% of the animals (31, 60). In the sensitive mouse model, the LD₅₀ of spores of STI-1 or Sterne is 10⁵ to 10⁶ (46, 58). We found that inoculation of guinea pigs with 10⁷ spores of the toxinogenic ATCC 14185 strain caused mortality of over 80% of the animals, while doses as high as 5 × 10⁸ to 1 × 10⁹ spores of MASC-10 and MASC-20 and their progenitor nontoxinogenic Δ14185 (Table 1) could be used safely to immunize guinea pigs (Table 4). In the mouse model, the LD₅₀ of ATCC 14185 was similar to that reported for Sterne or

TABLE 4. Efficacy of different *B. anthracis* vaccine strains following single immunization^a

Immunization		Anti-PA antibodies (GMT)		Protection ^b			
Strain	Dose	ELISA	Neutralizing	%	No. live/no. total	MTTD (days)	P
MASC-10	5 × 10 ⁷	900	400	100	8/8		<0.0001
	5 × 10 ⁶	120	60	100	8/8		<0.0001
	1 × 10 ⁶	<50	<50	87	7/8	9.2	0.0002
MASC-20	5 × 10 ⁷	200	80	100	8/8		<0.0001
	5 × 10 ⁶	<50	<50	75	6/8	9.0	0.0009
	1 × 10 ⁶	ND	ND	12	1/8	4.5	0.065
ATCC 14185 ^c	5 × 10 ⁶	500	200	100	8/8		<0.0001
	1 × 10 ⁶	ND	ND	25	2/8	4.7	0.065
Δ14185	5 × 10 ⁷	<50	<50	25	2/8	5.0	0.065
Saline		<50	<50	0	0/8	3.3	

^a Guinea pigs were inoculated with the indicated dose of spores, and 4 weeks postimmunization animals were bled or challenged. ND, not determined.

^b MTTD, mean time to death. P value determined versus saline-treated animals.

^c At a dose of 10⁷, ATCC 14185 spores killed 80% of the animals.

FIG. 4. Comparison of the immune responses induced by MASC-10 and MASC-20. Animals were immunized on day zero with 5×10^7 spores of MASC-10 (light bar) or MASC-20 (dark bar). Antibody titers (GMT) were detected at the indicated times postimmunization. Anti-core antibodies are directed towards the *B. anthracis* vegetative cell extract of $\Delta 14185$ (see Materials and Methods).

STI-1, and again both MASC-10 and $\Delta 14185$ were completely nonlethal at a dose of 10^8 spores (the highest dose tested). These results demonstrate that all the $\Delta 14185$ -derived strains are highly attenuated.

Single immunization with 5×10^7 spores of either MASC-10 or MASC-20 provided full protection from lethal challenge of Vollum. Yet, at lower doses of immunization (5×10^6 and 1×10^6), MASC-20 is clearly less efficacious than MASC-10, as suggested by the difference in MTTD, in survival rates, and in the neutralizing antibody titers induced by these two strains (Table 4). The immunization with 5×10^6 spores of MASC-20 provided 75% protection, and the titer of anti-PA antibodies is below the detection level, while MASC-10, under these conditions, provided full protection and induced measurable levels of anti-PA antibodies. Interestingly, at this dose, protective immunity of the recombinant MASC-10 and that of the ATCC 14185 strains are quite similar. The results suggest that the differential protection induced by MASC-10 and MASC-20 correlates with the PA-specific antibody titers induced by the two recombinant strains. However, at a lower dose (10^6 spores), none of the strains tested exhibited measurable antibody titers, and yet MASC-10 provided significant (P value of 0.0009) protection against the challenge compared to MASC-20.

Based on the studies described above, experiments were designed to compare the longevity of the protective immunity conferred by the two recombinant attenuated strains (MASC-10 and MASC-20) following a single inoculation of 5×10^7 spores (Fig. 4). Three months postvaccination, with

FIG. 5. Longevity of immunity as a function of immunizing dose of MASC-10 spores. Guinea pigs were immunized with a single dose of either 5×10^7 , 1×10^6 , or 1×10^5 spores of MASC-10 or with PBS as control animals. At the indicated time intervals postimmunization, animals were challenged or used to determine the titers (GMT) of anti-PA antibodies by ELISA (PA/ELISA) or by neutralization assay (Neutra. Ab.). The survival rates (live/total) of control animals upon challenge were 0, 25, and 12% at 1, 5, and 12 months, respectively.

either vaccine, all animals survived the lethal challenge of Vollum spores. Antibody titers induced by the two vaccines were consistently higher 2 months postinoculation than those measured after 1 month. As noted before, specific anti-PA antibodies were consistently higher in animals immunized with MASC-10 as compared to MASC-20, yet, at any given time, anti-core antibodies induced by the two recombinant strains were comparable.

The longevity of immune response was evaluated with variable immunizing doses (1×10^5 to 5×10^7) of MASC-10. Results summarized in Fig. 5 demonstrate that the longevity of protective immunity is dose dependent. At the lowest dose of inoculant (10^5 spores), immunized animals behaved the same as the mock-immunized control animals. At the highest immunizing dose (5×10^7), all animals survived the challenge even 12 months postvaccination. It is important to note that the level of anti-PA antibody titers (ELISA and neutralization) did not decline during the 12-month period (Fig. 5).

Efficacy of immunization with spores versus vegetative cells was determined for MASC-10. Animals challenged 1 month postimmunization with 5×10^7 spores were fully protected, while 5×10^7 CFU of the vegetative cells provided 75% protection (none of the control animals survived the challenge). This difference between vegetative cells and spores becomes significant with time. Thus, 5 months after the immunization, spores still provide full protection, while vegetative cells can protect only 40% of the animals from the lethal challenge ($P = 0.0018$) (Table 5). We noted that this poor level of protection by the vegetative MASC-10 cells is similar to that conferred by spores of the strain $\Delta 14185$ devoid of the *pagA* gene. Five months postimmunization with 5×10^7 spores of $\Delta 14185$, 60% of the animals were found to be protected from challenge (data not shown). The superiority of spores compared to vegetative

TABLE 5. The efficacy of spore versus vegetative attenuated *B. anthracis* vaccines^a

Immunization	% Protection ^b (no. live/ no. total)	Immune response ^b (GMT)			
		Anti-PA		Anti-core ^c ELISA	Anti-exosporium ^d ELISA
		Neutralizing	ELISA		
MASC-10 spores	100 (8/8)	740	2,900	2,800	15,800
Vegetative MASC-10 ^e	37 (3/8)	130	600	800	1,600
PBS	0 (0/8)	<50	<50	<50	≤200

^a Guinea pigs were inoculated once with a dose of 5×10^7 CFU of either spores or vegetative cells of the indicated strain.

^b Determined 5 months postimmunization.

^c The antibodies are captured by antigens prepared from the cellular matrix of vegetative Δ 14185 cells.

^d The antibodies are captured by the antigens prepared from the soluble fraction of outer-layer matrix of Δ 14185 spores.

^e *P* value of spores versus vegetative cells is 0.0018.

cells correlates with the higher anti-PA-specific as well as anti-core antibody titers in spore-immunized animals (Table 5).

DISCUSSION

In this study, we describe the engineering of live attenuated vaccines producing recombinant PA. These vaccines are spore-forming, nontoxigenic, and nonencapsulated *B. anthracis* bacteria, derived from the low-proteolytic V770-NP1-R strain (ATCC 14185). Doses as high as 1×10^8 to 5×10^8 spores of these recombinant vaccines can be used safely in guinea pigs or even in the sensitive mouse model, while classical human and veterinary vaccine strains, such as STI-1 or Sterne, are lethal at much lower doses (59). The various recombinant strains were engineered to express, from multicopy vectors, different levels of rPA by manipulation of the transcription signals, a regulatory gene (*atxA*), or translation signals. While *AtxA* could enhance the transcription of the PA driven by the *pagA* promoter in *B. anthracis*, this native promoter, in either *B. anthracis* or *B. subtilis* backgrounds, yielded consistently lower levels of PA in vitro than those attained by the synthetic heterologous α -amylase promoter (Table 2). Expression driven by this heterologous promoter in either *B. subtilis* or *B. anthracis* allowed secretion of at least 100 μ g of rPA per ml, which is among the highest production levels reported to date. It is worth noting that, at least in vitro, the plasmid carrying the cassette of the α -amylase promoter and the *pagA* gene is quite stable. More than 80% of spores prepared from vegetative MASC-10 cells grown without selection for 10 to 12 generations maintained the kanamycin resistance gene and retained the ability to produce high levels of PA.

In view of the well-documented central role of the PA in eliciting protective immunity, we verified the authenticity of the secreted rPA which was cloned from the Sterne strain by various biochemical analyses, including MS (Table 3). The rPA was also compared to the native PA (purified from strain V770-NP1-R used in the United States for preparation of the cell-free PA human vaccine) in biological assays such as cytotoxicity to J774A.1 cells, lethality to rats, and edema in guinea pigs. In all these tests, the biological function of the recombinant product was indistinguishable from that of native PA. Likewise, the immunogenicity and protective immunity elicited by an alum-adsorbed rPA vaccine were found to be similar to those of an identical vaccine formulation based on native PA prepared from V770-NP1-R.

From the various recombinant bacillus strains generated, we selected the *B. anthracis* MASC-10 and MASC-20 strains for a more extensive evaluation, where expression of PA is driven from the α -amylase and the *pagA* promoters, respectively.

These recombinant vaccines and their progenitor nontoxigenic strain Δ 14185 were used to evaluate the effects of variations in PA production in conjunction with different immunizing doses, as well as of the bacterial phase (spore/vegetative), on vaccine efficacy. We have used as a challenge a moderate dose (20 LD₅₀) of Vollum, a *B. anthracis* strain which appears to be less virulent to guinea pigs than the Ames strain (39, 60). It was expected that such a moderate challenge would allow us to reveal subtle differences between vaccines or immunization protocols.

We find that at similar immunizing doses (5×10^7 spores), the anti-PA antibodies (ELISA or neutralizing) elicited by MASC-10 are about fivefold higher than those generated by MASC-20 (Table 4). Furthermore and consistent with this observation, when animals were immunized with a 10-fold-higher dose of MASC-20 (5×10^7) as compared to MASC-10 (5×10^6), the animals developed similar anti-PA specific antibodies. Since we have shown in vitro (Table 2) that the α -amylase promoter is 3- to 10-fold more potent than the *pagA* promoter in *B. subtilis* and *B. anthracis* backgrounds (Table 2), it is very likely that the greater potency of MASC-10 over MASC-20 in inducing anti-PA antibodies is due to a higher in vivo level of expression of PA driven by the constitutive α -amylase promoter in the MASC-10 strain. This conclusion receives further support from the observation that when identical immunizing doses of MASC-10 and MASC-20 are used, animals develop similar anti-core antibody titers (Fig. 4). Therefore, the better protective immunity conferred by MASC-10 should be a direct consequence of the higher anti-PA response it induces in animals, since in all other parameters MASC-10 and MASC-20 appear to be similar. Barnard and Friedlander have demonstrated in a recent study the correlation between level of anti-PA antibodies and protective immunity (4). In addition, we have shown (Reuveny et al., unpublished data) by active immunization with decreasing doses of alum-adsorbed PA, as well as by passive transfer of PA-specific antibodies, that guinea pigs can be fully protected from a lethal challenge of Vollum, provided that the titer of neutralizing PA antibodies in the circulation exceeds a certain threshold level. However, results of vaccination with low doses of MASC-10 (10^6) or of MASC-20 (5×10^6), and more significantly with Δ 14185 (Tables 4 and 5 and Fig. 4), demonstrate that significant protection can be achieved without any detectable antibody titers to PA. We may therefore conclude, as stated previously (31, 39, 47, 58), that while PA clearly contributes to protection, there is another antigen(s) of *B. anthracis* which may contribute in a significant manner to protective immunity against anthrax.

To examine whether some of the protective immunogens are associated with spore antigens, we compared the responses following vaccination of guinea pigs with identical doses ($5 \times$

10^7 CFU) of spores or vegetative cells of MASC-10. Surprisingly, in spite of the relatively high anti-PA antibody titers (ELISA and even neutralizing) induced by the vegetative cells, only 40% of the animals were protected against a challenge of spores of Vollum (Table 5). This low efficacy of the vegetative cells is even more striking in view of the fact that spores of $\Delta 14185$, a strain not producing PA, were at least as efficient in protection as vegetative MASC-10 cells. These results imply that spore antigen(s) may have a more important role in eliciting protective immunity than previously suspected. Consistent with this proposal, we find that while the titers of anti-core antibodies are relatively similar in animals immunized with vegetative cells or spores, the anti-exosporium antibody titers are much higher in animals vaccinated with spores: 16,000 and 1,600 in animals immunized with MASC-10 spores or vegetative cells, respectively (we note that $\Delta 14185$ spores generate titers of 19,000 against exosporium) (Table 5). The practical implication of this observation is that spore, rather than vegetative, vaccines may be more suitable for protection against a spore challenge (as in scenarios of exposure to inhalation anthrax as well as cutaneous anthrax). No less significant is the observation that the spore vaccine appears to be a better antigen-presenting vehicle for the PA antigen than vegetative cells. This is demonstrated by the fivefold-higher neutralizing anti-PA antibody titers or ELISA titers induced by similar doses of spores compared to vegetative cells (Table 5).

Finally, the results indicate that a single immunization with MASC-10 at doses of 5×10^7 spores generates a long and lasting immunity, with relatively high neutralizing anti-PA, as well as high anti-exosporium antibody titers (Fig. 5 and Table 5). This response is stably maintained for at least 12 months and provides efficient protection from lethal challenge. We believe, therefore, that MASC-10 represents a platform of a prototypic, safe, and efficacious recombinant vaccine for further development and evaluation against a variety of virulent *B. anthracis* strains.

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