A recombinant 63-kDa form of *Bacillus anthracis* protective antigen produced in the yeast *Saccharomyces cerevisiae* provides protection in rabbit and primate inhalational challenge models of anthrax infection

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Abstract

Infection by *Bacillus anthracis* is preventable by prophylactic vaccination with several naturally derived and recombinant vaccine preparations. Existing data suggests that protection is mediated by antibodies directed against the protective antigen (PA) component of the anthrax toxin complex. PA is an 83-kDa protein cleaved in vivo to yield a biologically active 63-kDa protein. In an effort to evaluate the potential of yeast as an expression system for the production of recombinant PA, and to determine if the yeast-purified rPA63 can protect from a lethal inhalational challenge, the sequence of the 63-kDa form of PA was codon-optimized and expressed in the yeast *Saccharomyces cerevisiae*. Highly purified rPA63 isolated from *Saccharomyces* under denaturing conditions demonstrated reduced biological activity in a macrophage-killing assay compared to non-denatured rPA83 purified from *Escherichia coli*. Rabbits and non-human primates (NHP) immunized with rPA63 and later challenged with a lethal dose of *B. anthracis* spores were generally protected from infection. These results indicate that epitopes present in the 63-kDa form of PA can protect rabbits and non-human primates from a lethal spore challenge, and further suggest that a fully functional rPA63 is not required in order to provide these epitopes.

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1. Introduction

Anthrax, a disease caused by the spore-forming bacterium *Bacillus anthracis*, is highly lethal in the inhalational form...
**ABSTRACT**

Infection by Bacillus anthracis is preventable by prophylactic vaccination with several naturally derived and recombinant vaccine preparations. Existing data suggests that protection is mediated by antibodies directed against the protective antigen (PA) component of the anthrax toxin complex. PA is an 83-kDa protein cleaved in vivo to yield a biologically active 63-kDa protein. In an effort to evaluate the potential of yeast as an expression system for the production of recombinant PA, and to determine if the yeast-purified rPA63 can protect from a lethal inhalational challenge, the sequence of the 63-kDa form of PA was codon-optimized and expressed in the yeast Saccharomyces cerevisiae. Highly purified rPA63 isolated from Saccharomyces under denaturing conditions demonstrated reduced biological activity in a macrophage-killing assay compared to non-denatured rPA83 purified from Escherichia coli. Rabbits and non-human primates (NHP) immunized with rPA63 and later challenged with a lethal dose of B. anthracis spores were generally protected from infection. These results indicate that epitopes present in the 63-kDa form of PA can protect rabbits and non-human primates from a lethal spore challenge, and further suggest that a fully functional rPA63 is not required in order to provide these epitopes.
With an increased awareness of the potential of *B. anthracis* as a bioterrorist weapon, the need for a safe, effective, and readily available vaccine for wide public use has grown [7]. Vaccine preparations for human use have traditionally been attenuated live spores or cell-free secretion products of *B. anthracis* adsorbed to aluminum adjuvants [8,9]. The former preparation was used in the Soviet Union and was associated with a number of toxicities and production issues [10]. In the US, the latter is used exclusively and is currently sold as Biothrax™. Biothrax™ is a sterile culture filtrate from an attenuated non-encapsulated strain of *B. anthracis* adsorbed to an aluminum adjuvant, which contains unknown amounts of the three toxin components [10]. This vaccine requires a rigorous immunization schedule consisting of six doses over 18 months with an additional annual booster thereafter. Neither vaccine has the characteristics preferred for broad distribution in the event of an emergency, or for general public prophylaxis, i.e., long-term protection, high-level efficacy with a limited number of immunizations, low reactogenicity, and a reliable, reproducible means of production.

Although there are limited data in humans correlating protection against *B. anthracis* infection to specific immune responses, the available evidence suggests that antibodies directed against the 83-kDa form of PA (PA83) is a surrogate marker for protective immunity [11,12]. The data are more extensive in animal protection models using guinea pigs, rabbits, mice, and rhesus monkeys [13–15]. Immunization with purified native or recombinant PA3 confers protection against challenge in rhesus macaques [13], and passive protection was demonstrated in guinea pigs, mice, and rabbits with anti-PA3 antibodies [16–18]. There is a rough correlation between anti-PA3 titer and protection against lethal challenge with *B. anthracis* spores. Reunvey et al. [19] reported a more quantitative correlate with neutralization of in vitro macrophage killing for the guinea pig model. The cumulative evidence supports the concept that PA83, whether obtained from *B. anthracis*, or as a recombinant protein, can be an effective immunogen for anthrax prophylaxis.

Anthrax treatment and/or prophylaxis also show promise with mAbs to PA. In one report, mAbs to PA were generated by engrafting human peripheral blood lymphocytes from anthrax-vaccinated donors into severe combined immunodeficient (SCID) mice, followed by hybridoma formation and rescue of the genes encoding the protective antibodies [20]. Stable cell lines expressing full-length human immunoglobulin were subsequently created. These mAbs protected rats from a lethal dose of anthrax toxin in the Fisher 344 rat bolus toxin challenge model. More recently, a phase I clinical study was reported with PAmAb, a fully humanized mAb against PA that had demonstrated protection against challenge with *B. anthracis* spores in rabbit and monkey models of inhalational anthrax [21]. The mAb was safely administered to more than 100 healthy volunteers and shown to be well tolerated and bioavailable after either a single intravenous or intramuscular dose.

Published data show that the protective epitopes of PA83 can be found within the 63-kDa form of the protein [1,22–24]. There is some evidence that PA63 is also a protective immunogen. Ivins and Welkos cited a personal communication noting that immunization of guinea pigs with a 65-kDa fragment of PA83, produced by trypsinization, afforded protection against a challenge with virulent spores of the Sterne strain [25]. However, a replica construct designed to express the 63-kDa form of PA failed to protect immunized mice against challenge with spores from the Sterne strain, whereas a similar construct designed to express PA83 was protective [26]. Immunization of mice with DNA constructs designed to express either secreted or cellular PA63 (or PA83) conferred protection against challenge with anthrax toxin [27,28]. Gallo et al. showed that immunization with a PA63 construct followed by a boost with rPA83 afforded protection in a rabbit challenge model with spores of the Ames strain, but only one rabbit with this immunization regime was evaluated [29].

A variety of alternative vaccine preparations designed to minimize the occurrence of adverse experiences are currently being evaluated (for reviews see [8,9,27,30]). These include attenuated *B. anthracis* strains with enhanced PA3 production, recombinant protein products, and DNA-based vaccines. Containment issues related to using a live, attenuated strain of *B. anthracis* as a host may limit consideration of this system for large-scale production of PA, but recombinant production of toxin components appears to be a safe and viable alternative [8,9,30]. Production of rPA83 in *Escherichia coli* has been investigated extensively [30–35], and PA83 has also been expressed in transgenic tobacco chloroplasts [36]. An alternative expression system utilizing *Bacillus subtilis*, a related but non-pathogenic sporing *Bacillus* spp. was reported to have high endogenous protease activity that resulted in PA83 degradation [12].

*Saccharomyces cerevisiae* (Baker’s yeast) is generally recognized as an effective system for production of recombinant proteins, and antigens isolated from yeast have been shown to have good safety profiles. Examples of successful production in *S. cerevisiae* include recombinant hepatitis B surface antigen [37], and *human papillomavirus virus-like particles* [38,39]. In addition to safety, the *S. cerevisiae* expression system has a number of other benefits including ease of cultivation under conditions of minimal containment, and the ability to produce both small peptides and large proteins. Due to all of these features, yeast is being tested for production of a variety of additional antigens [40].

Based on the advantages of the yeast expression system for vaccine production, we wished to evaluate it for the production of recombinant PA. Here we present the design, expression, and purification of the 63-kDa activated form of PA, and demonstrate its’ protective efficacy in two independent animal models of inhalational anthrax infection.
2. Material and methods

2.1. Strains and media

*S. cerevisiae* strain 1260 (MATa, leu2, mnn9, ura3Δ, can1, his3Δ::GAL10-p-GAL4-URAS3, pfr1Δ::HIS3, cir′) was constructed using standard yeast genetic techniques. Strain 8-3 is a *Leu*⁺ transformant of 1260 designed to produce rPA63 intracellularly. Infectious anthrax spores were prepared from the *B. anthracis* Ames strain [41]. pET22b-PA-WT was obtained from Collier and co-workers [42]. The *Leu*⁺ yeast expression vectors pGAL110 [43] and pKH42 [44] were described previously.

Three different defined media lacking leucine were used for cultivation and fermentation of *S. cerevisiae*. SD-Leu medium was made from SD-Leu powder per the manufacturer’s instructions (QBIOgene, Carlsbad, CA). 5X minus leucine medium was described by Bayne et al. [45]. SDS-Leu medium contains 0.85% Yeast Nitrogen Base without added amino acids or ammonium sulfate, 1.0% succinic acid, 0.5% ammonium sulfate, 0.04% adenine, 0.03% l-isoleucine and l-phenylalanine, 0.025% l-tyrosine, 0.020% uracil, 1-tryptophan and l-lysine, 0.01% l-arginine, 0.005% l-methionine and l-histidine, adjusted to pH 5.3 with sodium hydroxide. YEHD is a complex medium and is comprised of YEHD [46] plus 4.0% (w/v) xylose and 0.85% (w/v) galactose. High-density medium is a chemically defined medium [47] optimized for cultivation of yeast strain 8-3. Trypsic soy agar was obtained from Difco (Franklin Lakes, NJ). Leighton and Doi medium [48] contains 16 g/L nutrient broth, 0.5 g/L magnesium sulfate, 2 g/L potassium chloride, 0.5 g/L magnesium sulfate, 0.2 g/L potassium chloride, 1 mM calcium nitrate, 0.1 mM manganese chloride, 1 µM ferric sulfate, and 0.1% glucose.

2.2. ELISA

rPA63 was quantitated in selected samples by sandwich ELISA utilizing monoclonal antibodies (MAbs) to PA63 for capture and detection (#C86301M and #C86613M, respectively, Biodesign International, Saco, ME). #C86301M was used at a final concentration of 5 µg/mL. C86613M was biotinylated with EZ-link™ Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacturer’s directions and used at a concentration of 1 µg/mL. Antibody binding was measured by dissociation-enhanced time-resolved flurometry with europium streptavidin (DELFIa En-N1 labeled streptavidin, Perkin-Elmer Life Sciences, Boston, MA) according to the manufacturer. Non-specific binding was determined by subtracting the value obtained with a cell lysate prepared from a vector-containing transformant, from that of an rPA63-expressing transformant. The amount of rPA63 was determined from a standard curve of rPA83 purified from *E. coli*. The sensitivity of detection of rPA83 was 10 ng/mL.

Sera were assayed for anti-PA antibody titers by direct ELISA. Ninety-six-well plates were coated with 100 ng/well of *E. coli*-expressed iPA83. Sera were serially diluted and transferred to the coated plates. Bound primary antibody was detected using an appropriate HRP-conjugated secondary antibody. Plates were developed using 3,3′,5,5′-tetramethylbenzidine (TMB) and end-point titers were determined using a cut-off of three times background.

2.3. SDS-PAGE and Western blotting

SDS-PAGE of process stream samples and final product was performed using either 10–20% Tris–HCl Criterion gels in 1X Tris glycine SDS buffer (Bio-Rad, Hercules, CA) or 4–20% NuPAGE gels with MOPS running buffer (Invitrogen, Carlsbad, CA). PVDF or nitrocellulose membranes were used for Western blots. Dot-blots were performed by diluting samples in 25 mM Tris, 0.15 M NaCl, pH 8.0 (TBS) containing 0.05% Tween 20 and applying to a nitrocellulose membrane using a Bio-Rad Biodot apparatus following the manufacturer’s directions. MAb C86613M was used as the primary antibody and goat anti-mouse-IgG horseradish peroxidase-linked whole antibody (Zymed Laboratories, South San Francisco, CA) was the secondary antibody. Blots were developed using the WESTERN LIGHTNING™ Chemiluminescence Reagent kit (Perkin-Elmer Life Sciences). Imaging was performed using X-ray film or a KODAK Imagenation 2000. Densitometry was performed on selected gels using KODAK 1D Image Analysis Software (version 3.6).

2.4. Purification of rPA83 expressed in *E. coli*

Recombinant PA83 purified from *E. coli* was used as a control or standard in various studies. The protein was expressed from pET22b-PA-WT in *E. coli* BL21 and purified as described previously with minor modifications [33]. Enzyme-generated PA63 (ipPA63) was derived from purified *E. coli* rPA83 by treatment with trypsin as described by Blaustein et al. [49] using trypsin (type XI) and soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). For certain studies, ipPA63 was purified by anion exchange chromatography on a Source Q column using a 0–1 M gradient of NaCl in 20 mM HEPPS, pH 8.2.

2.5. Construction and cloning of a yeast codon-optimized PA63 sequence

The gene fragment encoding ipPA63 was prepared using PCR in a step-wise fashion by the annealing and extension of 24 synthetic alternating and overlapping sense and antisense oligomers (91–110 bp in length) designed to encode the final desired sequence (the DNA sequence of the yeast codon-optimized ipPA63 was deposited in GenBank and assigned accession number DQ004463). Native Pfu polymerase (Stratagene) was used for the PCR in a “touch down” strategy as follows: 95°C, 90 s; one cycle; 95°C, 30 s; 55°C, 30 s, 68°C, 75 s for 5 cycles, immediately followed by a second series of reactions: 95°C, 30 s, 52°C, 30 s, 68°C, 75 s for 15 cycles. The reaction was completed by incubation at 68°C.
for 7 min. The final PCR used distal sense and antisense pri-
mers, 5′TGACGGAAGCACGTCTTCTTGGGATAAGAGATCCC-
ACCTCGG′ and 5′CGGGGATCCATAACCCATTCCCCTTCTGAT-
ACCCCTC′, respectively, which incorporated BamHI sites (underlined). The sense primer included additional upstream sequences to permit in frame cloning with the yeast alpha factor (MFα1) pre-pro secretory leader (in vec-
tor pKH4α2), and encoded the last seven amino acids of the pro-leader including its Lys-Arg KEX2 cleavage site. The resulting 1.7-kb product was gel-isolated and cloned into pCR-Blunt II-TOPO (Invitrogen) and then subcloned into the BamHI site of pEGEM3+ (Promega, Madison, WI). Sequence errors were corrected sequen-
tially by site-directed mutagenesis using the Quick-Change Site-directed Mutagenesis Kit (Stratagene) per the man-
ufacturer’s recommendations. A final PCR with High Fidelity PCR Supermix (Invitrogen) using an antisense primer 5′CGCCGGAACCTCTAAACATTGGTCAACCCCTT-
CTTAGAGAA3′, which incorporated a stop codon upstream of a BamHI site, and a sense primer which contained a BamHI site (5′-GTCTACCG ATC CTGTCT TGT AAG AGA-3′) amplified the corrected sequence (BamHI sites are underlined). The reaction product was subcloned into a TA cloning vector (Invitrogen) to construct pMK1.

For secretion of iPAA3, the BamHI fragment from pMK1 was subcloned downstream of the GAL10 promoter into the 
BamHI site of yeast secretory vector pKH4α2. The resul-
tant plasmid, pAGT1, contained an in frame fusion of the coding sequence of iPAA3 to the pre-pro-alpha factor leader sequence. To construct a plasmid for intracellular expres-
sion, the DNA encoding the iPAα3 protein carried in pMK1 was ampliﬁed with a sense primer that introduced a non-
translated leader sequence and ATG codon, and an antisense primer containing TAA as stop codon. The primers were 
5′GGGCGATCCCAAACAAAAATG TCCACCTCCGG-
CTGGTCCACGTGTTCC′ and 5′GGGGATCCATTAA-
CAATTTGGTACACCCCTTCTGAAAGA-3′, respectively, and both incorporate BamHI sites (underlined). The BamHI fragment was cloned into the BamHI site of pGAL110 under control of the S. cerevisiae GAL1 promoter to construct pPA63GAL110.

2.6. Cultivation of yeast transformants

Leu+ transformants containing iPAA3 were obtained with 
pPA63GAL110 and pAGT1 by a spheroplasting protocol 
[50] and selected on SD-Leu agar medium containing 1 M sorbitol. Small-scale trial cultivations of multiple yeast trans-
formants were conducted in 5 mL of broth in culture tubes. Cultures were incubated at 30 °C in SDC-Leu medium con-
taining 4.0% glucose and 0.1 M sorbitol for 18–24 h to an 
OD600 of 1.5–3.0/mL. A 0.3 mL aliquot was transferred to either 5.0 mL of SDC-Leu medium containing 2% glucose and 4% galactose or YEHDG medium. The cultures were incubated with slow rotation on a cell culture roller drum for 72 h to a final OD600 of 5–16.0/mL. A total of 10 ODs per sample were harvested for analysis. Cell pellets were col-
lected by centrifugation, washed once with PBS, and broken 
with 0.6 g glass beads (400–625 μm, Sigma Chemical CO, St. Louis, MO) in 0.3 mL lysis buffer (0.1 M sodium phos-
phate buffer, pH 7.2, 0.5 M sodium chloride, 1 mM PMSF). The lystate was recovered by centrifugation, the unbroken cells/beads were washed with an additional 0.3 mL of lysis buffer and the clarified supernatants were combined. The clari-
fied lysate was assayed for protein by the method of Bradford [51] with the Bio-Rad Protein Assay using the manufacturer’s instructions. Secretion of iPAA3 was evaluated directly from medium supernatants collected after centrifugation to remove the yeast cells. A variety of transformants and culture condi-
tions (including media and time dependence) were evaluated by this method. Subsequently, 50 mL cultures of several high-
producing transformants were cultivated in 250 mL shake 
flasks at 220 rpm. The transformant providing the highest level of expression was carried forward for scale-up in a fer-
mentor.

For fermentation at the 20 L-scale, a frozen stock of the 
transformant selected from the 50 mL cultures (strain 8-3), 
was thawed and 1.0 mL was inoculated into a 250 mL Erlen-
meyer flask with 50 mL of 5X Leu-medium containing 4% 
glucose. The flask was incubated at 28 °C, 250 rpm on a rotary 
shaker. After 24 h (residual glucose 22.3 g/L), a culture vol-
ume of 11 mL was added to a 2-L flask containing 877 mL of 
the same medium. Again, the flask was incubated at 28 °C and 
agitated at 250 rpm. After 24.5 h, the contents of the 2-L flask 
were used to inoculate a 20-L reactor containing high-density 
culture medium. This medium also contained 20 g/L glucose 
followed by 40 g/L galactose for induction. The reactor was 
operated at 28 °C, 4.7 L/min, 15 psig, and 300 rpm. Under 
these conditions, dissolved oxygen levels were maintained at 
greater than 30% saturation. Culture growth was monitored 
by glucose consumption, optical density at 600 nm, dry cell 
weight, galactose utilization, and ethanol production. Culti-
vation continued for 90 h reaching an optical density of 35 
and a dry cell weight of 19.7 g/L. The culture was harvested 
by hollow fiber tangential flow filtration using a 0.1 μm cut-
of membrane. Permeate was discarded and the cells were 
concentrated to 1.35 L, diafiltered with PBS, and collected 
by centrifugation at 10,800 × g, 4 °C for 20 min. The cell 
pellets were stored at −70 °C until needed.

2.7. Purification of yeast iPAA3

Frozen yeast cells were thawed in a cold-water bath and 
re-suspended in a 5× volume of cold breaking buffer: 0.2 M 
HEPES, pH 7.4, 2 mM MgCl2, containing 0.5 mM phenylmethylsulfonyl fluoride (Roche Applied Science, Indianapolis, IN), 1× “Complete” EDTA free protease inhibitor mix (Roche Applied Sci-
ence), 3.5 μg/mL pepstatin (Roche Applied Science), 20 mM benzamidine (Sigma-Aldrich Chemicals, St. Louis, MO). 
Benzonase™ (EMD Biosciences, San Diego, CA), 1 μL/g wet 
cell weight, was added and the cells were disrupted by four 
passes through a microfluidizer apparatus (Microfluidics,
Model 110S, Newton MA) at a pressure of 14,000 psi. The temperature of the lysate was maintained at 4 °C through- out the procedure, and the pH was maintained at 7.4 without adjustment. EDTA and EGTA were added to the lysate to final concentrations of 5 mM each. Insoluble material was removed by centrifugation (10,000 × g) for 20 min at 4 °C. The supernatant was clarified by filtration through a series of 47 mm filters (AP15, AP25, and 5 μM (Durapore White Hydrophilic Disc SVLP, Millipore)), and the clarified lysate was divided into aliquots for storage at −70 °C.

Frozen clarified lysate (320 mL) was thawed and adjusted to 6 M urea by addition of solid reagent (Pierce Biotechnol- ogy, Rockford, IL) and all chromatographic steps were performed at 2–8 °C. Denatured lysate was applied to a 1 L POROS 50RS (Amersham Biosciences, Piscataway NJ) cation exchange column equilibrated in 20 mM HEPES, pH 7.3 containing 6 M urea at a flow rate of 35 mL/min using a Waters 650 Prep system with a Linear UV–vis detector oper- ated at 280 nm. The column was washed with buffer until the signal returned to baseline. Elution was accomplished using a gradient of 0–1 M NaCl in equilibration buffer. Total pro- tein in column fractions was determined using a commercial bicinechonic acid (BCA) assay (Pierce, Rockford, IL), and fractions were analyzed by dot-blots, SDS-PAGE, and West- ern blots.

dPA63-containing fractions were pooled and concentrated over a 10-kDa cut-off tangential flow membrane (Millipore PrepScale TFF). The concentrated cation exchange pool was diluted 5-fold with 6 M urea in 20 mM HEPES buffer (pH 7.3) and applied over two runs to a 50 mL Source Q (Amersham Biosciences, Piscataway NJ) anion exchange column equilibrated in 20 mM HEPES, pH 7.3 containing 6 M urea at a flow rate of 20 mL/min. Elution was achieved using a gradient of 0–1 M NaCl in equilibration buffer and dPA63-containing fractions were pooled and dialyzed extensively against 20 mM HEPES, pH 7.3 containing 0.15 M NaCl. Six- teen micromolar CaCl2 was added to the refolding buffer based on studies showing a role for calcium in the folding of PA [52]. The anion exchange pool was sterile-filtered at 0.2 μm and stored in aliquots at −70 °C.

2.8. Biochemical characterization

The fluorescent dye 2-(p-toluidinyl)naphthalene-6-sulfonic acid (TNS) [53] was used to assess the relative hydrophobicity of the various purified forms of PA. Concentrated samples of yeast rPA63 or rPA63 were diluted into a stock solution of 50 μg/mL TNS (Molecular Probes, Eugene OR) in 0.1 M HEPES, pH 7.3, 0.15 M sodium chloride, 1 mM EDTA. The fluorescence spectra were obtained from 370 to 500 nm using an excitation wavelength of 466 nm in a Perkin-Elmer LS 50B Luminescence Spectrometer. The spectrum for TNS alone was used as a blank [54,55].

Yeast rPA63 was analyzed by circular dichroism (CD) using a JASCO J-810 spectropolarimeter. Purified rPA63 at 1 mg/mL in 20 mM HEPES, pH 7.3 was diluted 5-fold in 10 mM sodium phosphate, pH 7.4. Spectra were obtained at 25 °C from 260 to 185 nm and averaged over 10 scans. Quan- titative amino acid analysis was used to accurately determine the protein concentration of the CD samples, and the raw data were analyzed using CDPro software [56].

Mass determination was performed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on either intact rPA63 or rPA63 cleaved chemically with cyanogen bromide (CNBr), or enzymatically with EndoLyS-C (LysC). For intact protein and CNBr digestion analysis, an aliquot of the mixture was spotted onto target plates coated with an ultrathin layer of the matrix [57]. For analysis of Lys-C products (low or high resolution MALDI), the digest-matrix solution was spotted using the conventional dried-drop method and the spectra were externally calibrated with a standard peptide mixture. All measurements were made on a Reflex III mass spectrometer (Bruker Daltonics, Billerica, MA) operated in either positive ion linear mode (intact and CNBr digestion) or reflector mode (EndoLyS- C digestion). A Voyager-DE MALDI-TOF-MS instrument (Applied Biosystems, Foster City, CA) operated in linear mode was also used for EndoLyS-C digestion analysis.

2.9. In vitro and in vivo studies

To assess biological activity of the recombinant PA forms, a modification of a procedure based on the killing of macrophage cells by PA-LF toxin was used [58]. Two-fold serial dilutions of purified rPA63 or rPA83 (the latter expressed in E. coli) were incubated with 25 ng/mL rLF and 8 × 104 macrophage RAW264.7 cells/well in 96-well plates at 37 °C for 4 h. MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, (Promega)) and PMS (phenazine methosulfate, Promega) were added to final concentrations of 333 μg/mL, and 25 μM, respectively, and incubation continued for 2 h at 37 °C. Cell viability was then determined by measuring the absorbance at 490 nm. Maximum macrophage viability was assessed in the absence of LF-PA. The ability of sera to neutralize toxin was performed by pre-incubation of a lethal concentration of the rPA83/rLF toxin (50 ng/mL rPA83 and 15 ng/mL rLF) with 2-fold serial dilutions of various serum samples (beginning at 1:100) for 1 h prior to adding the mixture to the cells. The assay was completed as described above. The EC50 (50% effective concentration) corresponds to the dilution of antiserum that resulted in 50% neutralization.

All animal procedures were conducted according to USDA regulations and were approved by both the Merck and USAMRHD Institutional Animal Care and Use Committees. New Zealand White rabbits, 1.5–3.5 kg, were obtained from Covance Research Products (Denver, PA). Juvenile male and female thomus macaques, 1.5–2.5 kg, were obtained from the Merck NHP colony.

rPA63 was formulated for immunization by adsorption onto a proprietary Merck aluminum adjuvant (MAA) con-
3.1. Codon-optimized rPA63 expression in yeast

Analysis of the wild-type sequence for the gene encoding PA63 from B. anthracis Sterne strain (accession no. M22589 [62]) revealed that the codon usage of the bacterial gene differed significantly from that of genes known to express well in S. cerevisiae. Therefore, a synthetic PA63 gene sequence was constructed containing the same amino acid sequence encoded by the bacterial gene, but selected for yeast codons employed in highly expressed genes [63]. We evaluated both intra and extracellular expression of rPA63 in S. cerevisiae since it is difficult to predict a priori, which will be the most efficient means to produce a given heterologous protein. The synthetic PA63 gene was expressed under control of the inducible GAL1 or GAL10 Saccharomyces promoters from constructs designed for either intracellular expression or secretion, respectively. Transformants were obtained in four different yeast strains containing mnn9 and prb1 mutations to eliminate hypermannosylation of N-linked glycosylation and reduce proteolysis, respectively [64]. Galactose induction of expression was achieved by an auto-induction protocol as detailed in Section 2 and production of rPA63 was determined by Western blot analysis.

We evaluated extracellular production first because B. anthracis PA is secreted, and we thought it had good potential to be secreted by S. cerevisiae. Small-scale evaluation of PA expression from numerous transformants (seven per each yeast strain) revealed that each produced an immunoreactive protein of ~75 kDa, which was 12-kDa larger than expected, based on comparison with tPA63 (data not shown). No cross-reactivity of the antibody was obtained in supernatants from numerous vector control transformants tested. In addition, expression levels of the immunoreactive protein were less than 1 μg/mL in the culture medium.

In contrast, a protein that co-migrated with tPA63 was obtained from a construct designed for intracellular expression in all 28 transformants tested (of four different yeast strains; data not shown). All of these transformants displayed volumetric titers of at least 5 μg/mL rPA63 in YEHDG culture medium. The best transformant, in terms of yield and quality of rPA63 produced, was strain 8-3, a transformant of strain 1260. Cultivation in complex YEHDG medium for 72 h at 30 °C was found to be optimal for expression. Fig. 1, lanes 7 and 8, show a Western blot depicting two different proteins of cell lysate from strain 8-3, a transformant of strain 1260. Cultivation in complex YEHDG medium for 72 h at 30 °C was found to be optimal for expression. Fig. 1, lanes 7 and 8, show a Western blot depicting two different proteins of cell lysate from strain 8-3, a transformant of strain 1260. Cultivation in complex YEHDG medium for 72 h at 30 °C was found to be optimal for expression. Fig. 1, lanes 7 and 8, show a Western blot depicting two different proteins of cell lysate from strain 8-3, a transformant of strain 1260.
Fig. 1. Western blot depicting intracellular expression of rPA63 by strain 8-3. Lanes 1 and 2 represent 10 and 20 ng, respectively, of tPA63 mixed with 500 ng of total protein from the cell lysate of vector transformant 7-1. Lanes 3 and 4 represent 10 and 20 ng, respectively, of (rPA63) purified from E. coli, mixed with 500 ng protein cell lysate from vector transformant 7-1. Lanes 5 and 6 represent 250 and 500 ng, respectively, of cell lysate protein from 20-L fermentation (high density) of strain 8-3, while lanes 7 and 8 represent 250 and 500 ng, respectively, of cell lysate protein from small-scale cultivation of strain 8-3. 

protein was minimal. The cultivation conditions described above resulted in a volumetric productivity of 65 and 80 μg rPA63/mL as determined by Western blot and ELISA, respectively, with rPA63 comprising 8% of the total yeast protein.

Fermentation of strain 8-3 was conducted in high-density medium. The major species produced co-migrated with tPA63 (Fig. 1, lanes 5, 6). The average concentration of rPA63 in the high-density fermentation, determined by both Western blot and ELISA, was 288 ± 97 μg/mL (avg. ± 1 SD), and rPA63 represented 10–12% of the total yeast protein as determined by densitometry of SDS polyacrylamide gels stained for total protein (as shown in Fig. 2A).

3.2. Purification of rPA63

Initial attempts to purify yeast rPA63 under non-denaturing conditions were unsuccessful. The rPA63 was not significantly enriched in any particular fractions using either anion or cation exchange chromatography. This behavior was observed for multiple column types and did not appear to be associated with a particular resin or manufacturer. Suspecting that multimerization of rPA63 might be the reason for the broad elution spectrum, we attempted to isolate the protein under denaturing conditions, where it would be largely unfolded and thus represent a single species.

rPA63 was successfully isolated under denaturing conditions from fermentor-grown cells cultivated in high-density medium. Clarified yeast lysate was adjusted to 6 M urea and processed by cation exchange chromatography. rPA63 eluted from the column as a sharp peak at approximately 100–150 mM sodium chloride. Fractions were pooled based on UV absorbance, and dialyzed to remove denaturant. No protein precipitation was observed during dialysis. Subsequent sterile filtration of the dialyzed product pool did not result in any detectable loss of protein (recovery >99%). Purity of the final sterile-filtered pool was determined by densitometry and found to be greater than 90%. Recoveries of rPA63 for the major steps of the purification process are presented in Table 2. The yield of rPA63 through the purification process was approximately 2.5 g of rPA63/kg of cell paste, and the volumetric productivity of the...
Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>Protein (µg/mL)</th>
<th>Total Protein (mg)</th>
<th>Purity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clarified lysate a</td>
<td>320</td>
<td>13,200</td>
<td>4224</td>
<td>~17</td>
</tr>
<tr>
<td>Cation exchange pool</td>
<td>420</td>
<td>1000</td>
<td>420</td>
<td>~40</td>
</tr>
<tr>
<td>Anion exchange pool</td>
<td>240</td>
<td>549</td>
<td>141</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

* The clarified lysate was prepared from a starting wet cell weight of 55.8 g.

Fermentation was approximately 181 mg/L. Representative images of the results obtained by SDS-PAGE and Western blotting of the final product are shown in Fig. 2 B and C.

3.3. Biochemical characterization of rPA63

A mass of 63,494 ± 11 Da was obtained for rPA63 as determined by MALDI-TOF-MS. This mass compares favorably with the predicted mass of 63,491. Endo Lys-C digestion produced a fragment at m/z 3175.9, which agrees with the rPA63 sequence of residues 1–30 (N-Ac-STSAGPTVPDRDNDGIPDSLEVEGYTVDFK) at an expected m/z (monoisotopic M + H) of 3175.5. This data confirms acetylation of the N-terminal serine, and there was no indication of non-acetylated peptide in the spectrum. The MALDI peaks for rPA63 were narrow and well defined, and mapping the protein with Endo Lys-C and CNBr digestions yielded 100% sequence coverage, confirming that none of the amino acids were modified. The CD analysis of yeast rPA63 and tPA63 is shown in Fig. 3 A. The spectra of the two forms of PA63 are visibly different; with tPA63 showing a much more defined negative ellipticity near 220 nm, which would qualitatively suggest a higher alpha helical content. The minimum around 205 nm observed for tPA63 is suggestive of a more randomized structure. This observation is also consistent with the lower positive value of the ellipticity at 190 nm for rPA63 compared with tPA63. The spectra of tPA63 and tPA63 were analyzed by several programs (CDSSTR, SELCON3, and CONTINLL) used for estimation of secondary structure. The results of these analyses also suggest that yeast rPA63 has a higher degree of unordered structure than tPA63 (see inset in Fig. 3 A). Fig. 3 B shows the fluorescence spectra of rPA63 and tPA63 bound to the hydrophobic dye TNS. The higher observed intensity of yeast rPA63 at equivalent amounts of protein suggests that it has a higher hydrophobic character than tPA63.

3.4. Yeast produced rPA63 shows biological activity

The biological activity of yeast rPA63 was measured by its ability to combine with E. coli rLF and kill cultured macrophages. Fig. 4 shows that the purified yeast rPA63 was active in this assay, albeit at a higher concentration than control tPA63 purified from E. coli. E. coli rPA63 enabled 50% killing at a concentration of less than 10 ng/mL, while the rPA63 from yeast required approximately 1 µg/mL to achieve the same level of toxicity. The lower toxicity obtained with yeast rPA63 was not due to the presence of an inhibitor in the preparation as mixing experiments demonstrated that yeast rPA63 did not inhibit toxin activity when E. coli rPA63 and rLF were combined (data not shown). Neither yeast rPA63

Fig. 3. (A) CD Spectra of rPA63 (dashed line) and tPA63 (solid line). (B) TNS fluorescence of rPA63 (dashed line), and tPA63 (solid line).

Fig. 4. Toxicity of rPA63 (closed circles) when combined with rLF in the macrophage-killing assay. E. coli produced rPA63 (open circles) was included as a control. The closed and open squares depict rPA63 and tPA63, respectively, in the absence of LF.

Fig. 5. (A) Time course showing rabbit end point titers to rPA83 following immunization with rPA63 (50 \( / H9262 \) g, closed circles; 5 \( / H9262 \) g, open circles; 0.5 \( / H9262 \) g, closed triangles), BiothraxTM (closed squares), or an adjuvant control (open triangles). Immunizations are indicated by the inverted arrows in each plot. Note that the NHP 50 and 5 \( / H9262 \) g rPA63 titers overlap except for the final time point.

or E. coli rPA83 were observed to have any toxicity in the absence of rLF.

3.5. Purified rPA63 is immunogenic in rabbits and NHP

Rabbits and NHP immunized with the experimental yeast rPA63 vaccine mounted a vigorous response, producing IgG that was cross-reactive with rPA83 as shown in Fig. 5A and B, respectively. All rabbits and NHP in the test vaccine groups developed an immune response after two injections, and antibody titers continued to increase after the third injection.

Animals receiving BiothraxTM showed increased titers after a single injection that were boosted after the second injection, and by a third injection that was given to NHP only. Rabbits receiving three 50 \( / H9262 \) g rPA63 doses reached equivalent titers to rabbits receiving two injections of one fourth of the human dose of BiothraxTM (Table 3a). NHP receiving three 50 \( / H9262 \) g doses of rPA63 had geometric mean titers similar to the NHP receiving three doses of BiothraxTM (Table 3b). The geometric mean titer for the 5 \( / H9262 \) g rPA63-immunized primates was approximately 50% of the BiothraxTM and 50 \( / H9262 \) g rPA63 groups.

3.6. Anti-rPA63 sera inhibits LF-PA-mediated killing of cultured macrophages

Individual antisera (collected 1 week prior to challenge) from rabbits that were immunized with the third 50 \( / H9262 \) g dose of yeast rPA63 (10 animals), the second dose of BiothraxTM (5 animals), and the third dose of MAA (2 animals) were tested for the ability to neutralize anthrax toxin in the macrophage killing assay. Two-fold serial dilutions from 100 to 102,400 were performed for each antiserum +/− LF-PA. None of the antisera neutralized LF-PA.

Table 3a
Geometric mean titers and survival of rabbits immunized with rPA63, BiothraxTM, or MAA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>GMT±1</th>
<th>Survival</th>
<th>LD50±2</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ( / H9262 ) g rPA63</td>
<td>649,889</td>
<td>6 of 10</td>
<td>647 ± 216</td>
</tr>
<tr>
<td>5 ( / H9262 ) g rPA63</td>
<td>373,213</td>
<td>8 of 9</td>
<td>696 ± 184</td>
</tr>
<tr>
<td>0.5 ( / H9262 ) g rPA63</td>
<td>166,511</td>
<td>5 of 10</td>
<td>784 ± 268</td>
</tr>
<tr>
<td>BiothraxTM</td>
<td>799,882</td>
<td>5 of 5</td>
<td>600 ± 107</td>
</tr>
<tr>
<td>MAA</td>
<td>1</td>
<td>0 of 10</td>
<td>651 ± 20</td>
</tr>
</tbody>
</table>

1 Titers determined on blood collected 1 week prior to challenge.
2 Value represents the average ± 1 S.D.

Table 3b
Geometric mean titers and survival of rhesus macaques immunized with rPA63, BiothraxTM, or MAA

<table>
<thead>
<tr>
<th>Antigen</th>
<th>GMT±1</th>
<th>Survival</th>
<th>LD50±2</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ( / H9262 ) g rPA63</td>
<td>100,794</td>
<td>2 of 3</td>
<td>874 ± 692</td>
</tr>
<tr>
<td>5 ( / H9262 ) g rPA63</td>
<td>65,496</td>
<td>3 of 3</td>
<td>1350 ± 688</td>
</tr>
<tr>
<td>BiothraxTM</td>
<td>100,794</td>
<td>2 of 3</td>
<td>795 ± 243</td>
</tr>
<tr>
<td>MAA</td>
<td>1</td>
<td>0 of 3</td>
<td>1226 ± 283</td>
</tr>
</tbody>
</table>

1 Titers determined on blood collected 1 week prior to challenge.
2 Value represents the average ± 1 S.D.

Individual antisera (collected 1 week prior to challenge) from rabbits that were immunized with the third 50 \( / H9262 \) g dose of yeast rPA63 (10 animals), the second dose of BiothraxTM (5 animals), and the third dose of MAA (2 animals) were tested for the ability to neutralize anthrax toxin in the macrophage killing assay. Two-fold serial dilutions from 100 to 102,400 were performed for each antiserum +/− LF-PA. None of the antisera neutralized LF-PA.
the antiserum alone appeared to be toxic to the macrophages since no effect on viability was seen in the absence of LF-PA (data not shown). No inhibition of macrophage killing by LF-PA toxin was observed with MAA controls or pre-immune sera. Neutralization of toxin was obtained with antiserum from all rabbits immunized with yeast rPA63 and BiothraxTM as shown in Fig. 6. The mean EC₅₀ for the rPA63 group (Table 4) was approximately seventy percent that of the Biothrax group (1:1361 versus 1:1968, respectively), although this difference did not reach statistical significance (Mann–Whitney rank sum (p = 0.076)) [65].

3.7. rPA63 confers protection in two experimental models of inhalational infection

The rabbits in this study were challenged with approximately 675 LD₅₀ spores of B. anthracis Ames strain at 4 weeks after the last injection of rPA63 or MAA, and 3 weeks after the last injection of BiothraxTM. Survival of the rabbits receiving rPA63 or BiothraxTM was higher than the MAA controls as shown in Table 3a. All of the animals that received MAA died, whereas 19 of 29 rabbits receiving rPA63, and five of five receiving BiothraxTM survived. All doses of rPA63 showed equivalent protection (p > 0.15). Survival of rabbits in the immunized groups was significantly greater than the adjuvant control group (p < 0.01), and similar to survival in the group of rabbits given BiothraxTM.

NHP were challenged with approximately 1000 LD₅₀ spores of B. anthracis Ames strain. A survival plot for the non-human primate challenge is shown in Fig. 7. Survival of the monkeys vaccinated with rPA63 or BiothraxTM appeared significantly greater than the adjuvant-controls although there were not enough animals in the study to assign a p-value (Table 3b). The three control monkeys that received adjuvant alone died on days 4, 5, and 6 post-challenge. One of the three monkeys that received 50 μg rPA63 and one of the three that received BiothraxTM died on days 5 and 8 post-challenge, respectively. All of the remaining monkeys survived and showed no signs of illness as of day 21 post-challenge.

4. Discussion

This study was initiated after the 2001 anthrax attacks in order to determine the feasibility of producing a PA-based vaccine in S. cerevisiae. Our rationale for expressing the 63-kDa form of the antigen was: (a) interest in extracellular production which was thought to be more achievable with the mature protein; (b) concern that intact PA83 would be difficult to isolate due to endogenous yeast protease activity; (c) literature reports suggesting that protective epitopes were present on PA63[1,22–24]; and (d) the potential that PA63 might contain additional protective epitopes generated by conformational changes resulting from protease cleavage of the 83-kDa protein. Indeed both PA63 monomer and PA63 heptamer/LF complexes have been detected in the blood of infected animals, whereas PA83 has not[66,67]. S. cerevisiae was chosen based on in-house experience with this expression system for vaccine manufacturing, and because of the excellent safety profile of currently licensed and experimental yeast-derived vaccines such as RecombivaxTM [68,69], and GardasilTM [38].

The current state of understanding of protein production in yeast does not allow one to predict which proteins will be made in sufficient quantities for commercial production. The degree of glycosylation, extent of protein degradation, and correct polypeptide folding are all variables that must be determined empirically for a particular recombinant protein. Because PA is a protein that is secreted naturally by B. anthracis, we first tried to express rPA63 as a secreted extracellular protein in S. cerevisiae to facilitate downstream purification. Several difficulties were encountered in this line of experimentation. PA63 produced via the secretory pathway...
migrated in SDS-PAGE gels at an apparent molecular weight of 75 kDa, which is approximately 12,000 Da higher than the predicted molecular weight for the activated form. The increase in mass could have resulted from N-glycosylation since rPA63 contains eight potential Asn-X-Seq/Thr consensus sequences. Although the rPA63 construct was expressed in minE9 mutants to prevent elongation of N-linked glycosylation, these mutations do not preclude core glycosylation. There are also numerous potential sites for O-glycosylation, which is even more difficult to predict. Alternatively, the higher molecular weight could be due to improper cleavage of the alpha factor pre-pro leader sequence, which was used to direct the protein to the secretory pathway. Expression of proteins in S. cerevisiae pep4 mutants sometimes improves cleavage of lys-arg-containing leader sequences (like alpha factor) by Kex2 protease, since Kex2p is a substrate of Pep4 [40]. However, the molecular weight of rPA secreted in pep4 mutants was the same as in strains that contained wild-type PEP4 (data not shown). The alpha factor pre-pro leader amino acid sequence in vector pH4n2 differs from the authentic leader by two amino acids due to the introduction of a convenient cloning site [44]. Expression of rPA63 in a different secretory vector, pH41B [46], containing an authentic alpha-factor pre-pro leader, did not improve either the quality or quantity of rPA63 secreted (data not shown). A major disadvantage to extracellular expression was that very low volumetric productivity was obtained under numerous fermentation conditions, despite the fact that the gene sequence was codon-optimized for S. cerevisiae. Future studies are needed to discern whether rPA63 is indeed glycosylated and/or contains uncleaved leader sequence in order to optimize secretion of rPA63 in yeast.

In contrast to our attempts to secrete rPA63, we were successful in achieving high-level expression of rPA63 intracellularly. The highest specific productivity was obtained in a protease and glycosylation-deficient strain enhanced for over-expression of the Saccharomyces GAL4 transcription factor. Intracellular production of rPA63 scaled-up favorably from shake-flasks to fermentors in terms of both specific content, and percent of total protein. There was no evidence of significant proteolytic degradation of rPA63 in either small-scale cultivations (tub or flask), or 20-L fermentations.

Purification of rPA63 provided an unanticipated challenge. Isolation of the protein under native conditions was not achieved. A variety of anion and cation exchange matrices were evaluated under non-denaturing conditions, and in each case the product bled slowly off of the column instead of eluting as a defined peak. A possible explanation for this behavior rests with the oligomerization-prone nature of PA63. In vivo, receptor-bound PA63 self-associates to form heptamers capable of binding LF and/or EF. In yeast, the structural characteristics of the protein responsible for self-association may result in production of multiple oligomeric forms of PA, or formation of PA—yeast protein complexes intracellularly or during early stages of the purification process, thus, preventing efficient isolation under native conditions.
made in the NHP model as the time between the final boosts and challenge differed due to an unanticipated scheduling conflict after the experiment had been initiated. In rabbits, all doses of rPA63 (50, 5, and 0.5 µg) and Biothrax™ showed equivalent protection, and survival in all immunized groups was significantly greater than in the adjuvant control group. The protection obtained in the NHPs is noteworthy as they were exposed to a spore challenge dose that was five times greater than the established LD50 for this species. Nevertheless, five of six animals immunized with either 5 or 50 µg of rPA63 survived, as did two out of the three NHP that received Biothrax™. Statistical analysis of the primate data was not warranted due to the small sample sizes involved, however, the ability of rPA63 to protect against anthrax infection does compare favorably with a prior study demonstrating protection by rIPA63 in rhesus macaques [73]. The data are also in agreement with a communication describing protection against challenge with infectious spores of the B. anthracis Sterne strain by immunization of guinea pigs with IPA63 [25].

The question of whether rPA63 has any advantages relative to rPA83 as a vaccine antigen was beyond the scope of this study and is a topic for future study. However, rPA63 seems advantageous for expression and purification in yeast. We did express codon-optimized rPA83 intracellularly in S. cerevisiae and found that higher yields were obtained with rPA63, and the rPA83 tended to be more sensitive to the presence of yeast proteases (data not shown).

Herrman et al. found that a cationic lipid-formulated rPA63 (lacking the furin cleavage site) plasmid DNA vaccine was more efficacious than a similar rPA63 construct with respect to immunogenicity in mice and the ability of mouse antisera to neutralize Leth [74]. The rPA83 construct was further evaluated and shown to protect rabbits against inhalational challenge with spores from the Ames strain. Greater survival of the rabbits was obtained by immunization with rPA63, and the rPA83 tended to be more sensitive to the presence of yeast proteases (data not shown).

This work supports the results of previous studies by Ramerez et al. [71], which demonstrated that recombinant PA83 altered to prevent protease activation and heptamerization did generate high levels of IgG with toxin-neutralizing activity. However, our study is the first to demonstrate that a completely functional PA is not required for protection against anthrax infection in a NHP inhalational challenge model. An inactive form of rPA83 referred to as DNI that a completely functional PA is not required for protective immunity. Med Microbiol Immunol 1988;177(5):293–303.


Acknowledgements

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