Identification of novel and cross-species seroreactive proteins from *Bacillus anthracis* using a ligation-independent cloning-based, SOS-inducible expression system

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Abstract

The current standard for *Bacillus anthracis* vaccination is the Anthrax Vaccine Adsorbed (AVA, BioThrax). While effective, the licensed vaccine schedule requires five intramuscular injections in the priming series and yearly boosters to sustain protection. One potential approach to maintain or improve the protection afforded by an anthrax vaccine, but requiring fewer doses, is through the use of purified proteins to enhance an antibody response, which could be used on their own or in combination with the current vaccine. This study describes a novel, high-throughput system to amplify and clone every gene in the *B. anthracis* pXO1 and pXO2 virulence plasmids. We attempted to express each cloned gene in *Escherichia coli*, and obtained full-length expression of 57% of the proteins. Expressed proteins were then used to identify immunogens using serum from three different mammalian infection models: Dutch-belted rabbits, BALB/c mice, and rhesus macaque monkeys. Ten proteins were detected by antibodies in all of these models, eight of which have not been identified as immunoreactive in other studies to date. Serum was also collected from humans who had received the AVA vaccine, and similar screens showed that antigens that were detected in the infection models were not present in the serum of vaccinated humans, suggesting that antibodies elicited by the current AVA vaccine do not react with the immunoreactive proteins identified in this study. These results will contribute to the future selection of targets in antigenicity and protection studies as one or more of these proteins may prove to be worthy of inclusion in future vaccine preparations.

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Keywords

Bacillus anthracis; recombinant vaccine; ligation-independent cloning; high-throughput expression; seroreactive

1. Introduction

Bacillus anthracis is a Gram-positive soil-borne bacteria and the causative agent of anthrax. Natural human anthrax infections are typically acquired by handling animal products contaminated with *B. anthracis* spores and normally present as a reasonably treatable cutaneous infection. Other forms of anthrax include gastrointestinal anthrax resulting from the ingestion of a contaminated animal product, and inhalational anthrax, which is the most deadly form of the disease and progresses very quickly [1,2]. The lethal potential of *B. anthracis* spores combined with their hardiness and ease of preparation has made them a central component to biological weapons research over the past 60 years in multiple countries, including Japan, the former Soviet Union, and Great Britain [3-5]. The anthrax letter attacks in the United States in 2001 resulted in 22 cases of inhalational anthrax, five of which were fatal even after intense antimicrobial therapy, demonstrating the potential use of this agent as an instrument in a future biological attack [6].

Two plasmids, pXO1 and pXO2, are maintained by virulent *B. anthracis* and impart much of this pathogen's virulence. The 184.5 kb pXO1 plasmid encodes the tripartite toxin complex [lethal factor (LF), protective antigen (PA) and edema factor (EF)], all of which are required for full virulence [7]. The 95.3 kb pXO2 plasmid is required for the synthesis of the capsule proteins responsible for inhibiting phagocytosis of *B. anthracis* spores [7,8].

In the United States, the only FDA-licensed vaccine against anthrax is the Anthrax Vaccine Adsorbed (AVA [BioThrax]; Emergent BioSolutions, Lansing, MI). This vaccine is composed of aluminum hydroxide-adsorbed culture supernatant, with the primary protective component being *B. anthracis* protein PA [9]. This vaccine is known to protect against inhalational anthrax in multiple animal models and in humans [10-12]. However, the regimen for this vaccine is somewhat cumbersome and expensive. It consists of a series of doses administered at 0 and 4 weeks, and at 6, 12, and 18 months, with yearly boosters [13]. Additionally, evaluation of the safety of the AVA vaccine is still ongoing. Multiple studies have shown that the protein composition of the vaccine varies from lot to lot, and other *B. anthracis* components, including the lethal factor toxin, are known to be present in ambiguous amounts [14,15]. It has also been demonstrated that the vaccine can cause some systemic and local reactions, including headache, fever, and injection site sensitivity [16]. As a result of these issues, there is a current effort to design a vaccine that displays increased safety and efficacy while sustaining or surpassing the protectiveness of the AVA vaccine.

Since PA is known to be the primary protective component of the AVA vaccine, efforts are underway to explore the use of recombinant PA as the active component of a new vaccine. Initial studies in rabbits [17] and nonhuman primates [18] showed a high level of PA-mediated protection against aerosol infection, and Phase I trials indicated that while recombinant PA is safe, important features such as optimal formulation and dosing schedule require further development [19,20]. Other studies have sought to combine the purified, recombinant PA with other *B. anthracis* proteins that elicit protective responses and could thus enhance the protection afforded by PA alone. Such "cocktail" style vaccines have seen a great deal of success against *Bordetella pertussis* infections. Pertussis vaccines licensed in the United States combine inactivated pertussis toxin with other bacterial components, including filamentous hemagglutinin, pertactin, and fimbrae proteins. One study

demonstrated that acellular pertussis vaccines increased in efficacy as the number of recombinant protein components increased from one to three or more [21].

The success of the *B. pertussis* cocktail vaccine has contributed to the effort to identify antigenic *B. anthracis* proteins that may be used as components of a cocktail vaccine against anthrax infection [22-30]. One series of experiments combined PA with poly- -d-glutamic acid from the capsule to enhance protection against a spore challenge in mice and rabbits [31,32]. Other experiments have explored the use of spore-associated proteins in combination with PA. One such protein, BclA, was shown to afford a higher level of protection than PA alone in mice [27]. Additionally, the two spore proteins p5303 and BxpB were also shown to improve protection in mice after spore challenge when given in combination with PA [33].

Publication of the *B. anthracis* genome has paved the way for more focused efforts to identify and characterize potential candidates for vaccine studies. Computational analysis of the *B. anthracis* proteome has led to the identification of potentially immunogenic proteins that were assessed in a DNA vaccination study [34]. In addition, *in vivo* Induced Antigen Technology (IVIAT) strategies have identified seroreactive proteins that are expressed only under conditions similar to those seen during an infection [22].

Previous work has identified many seroreactive proteins within the *B. anthracis* proteome, and some of the genes encoding these proteins are found on the virulence plasmids (Table 1) [35]. Thus, we aimed to clone every virulence plasmid gene into an *Escherichia coli* expression vector and use a battery of sera from animal infection models to probe proteins expressed from this clone set for infection-specific seroreactive proteins. The novel vector created for use in this study combines the efficiency of Ligation-Independent Cloning (Stratagene) with the ColE1 promoter to attempt to clone and express the 226 *B. anthracis* virulence plasmid genes in *E. coli*. Expressed proteins were used in immunoblots using serum from mouse, rabbit, and macaque infection models to test for seroreactivity. These proteins were also used in immunoblots using serum from five human AVA recipients in an attempt to identify other immunoreactive components that may contribute to the protection provided by the AVA vaccine. The identification of unique seroreactive proteins in this study has added to the list of candidate proteins identified in other studies to be investigated further for their ability to stimulate a protective immune response against *B. anthracis* challenge. Inclusion of promising candidates in future vaccine preparations may improve vaccine protectiveness and could help minimize the cost, risk, and time involved to sustain immunity.

2. Materials and Methods

2.1 Ligation-independent cloning of *B. anthracis* genes

The genes from which the pXO1 clone set was derived were originally sequenced from a pXO2-deficient Sterne strain ([36], AF065404), and the genes from which the pXO2 clone set were derived was from a Pasteur strain sequenced in 1999 and directly submitted to NCBI by Okinaka et. al. (AF188935). The *cea* promoter was amplified from the plasmid pGE124 [37] by PCR using primers HG3 and HG4 (Table 2). The *cea* promoter fragment and the pTRC99A plasmid were digested with NdeI and EcoRI (New England Biolabs) and ligated. This new construct, pHG2, was electroporated into JM109 cells.

The *tet* gene was then amplified from pKRP12 [38] using primers HG50 and HG52 (Table 2), and the product was digested with EcoRI and HindIII and ligated into pHG2 to create pHG4. This step also added a 10x histidine tag to the C-terminus of the *tet* gene. The *cea* promoter, 10xHis-tagged *tet* gene, and *rrnB* T1T2 transcriptional terminator fragment of

pHG4 were amplified using HG53 and HG54, digested with NdeI and SapI, and ligated into the same sites of the pUC18 plasmid to create pHG5. Addition of a T7 antigen tag upstream of the *tet* gene and ligation independent cloning (LIC) was achieved by amplifying the *tet* gene with HG55 and HG56 and replacing the pHG5 EcoRI-HindIII fragment with the HG55-HG66 PCR product. A NotI digest of pHG6 and recircularization of the plasmid backbone removed the *tet* gene from this construct, leaving the LIC cloning regions and flanking fusion tags. This final construct was named pHG7 (Figure 1).

For ligation independent cloning of pXOl and pX02 ORFs, the pHG7 plasmid was digested with NotI (Figure 2). Incubation with T4 DNA Polymerase and dTTP removed bases from the 3 overhangs to the first thymidine encountered. Primers designed to complement the gene of interest and the T4 DNA polymerase-generated overhang were used for PCR. The PCR product containing the gene of interest was subsequently exposed to T4 DNA polymerase and dATP, which removed bases on the 3 end of the overhang until the first adenosine. The two complimentary overhangs created by these steps were used to anneal the gene of interest into the pHG7 expression plasmid (Figure 1). Ligation of the resulting nicked insert is accomplished by the host machinery after transformation into JM109 cells. Each clone was verified by restriction digest and DNA sequencing.

2.2 Expression under low-oxygen conditions

Cultures of selected clones were grown overnight in 10 mL of Luria-Bertani broth supplemented with 0.15 mM KNO_3 and 100 μg/mL ampicillin. High aeration samples were diluted 1:100 into 50 mL fresh media in a 250 mL baffled flask and allowed to grow to an OD_{600} of   0.5. Protein expression was induced with 1 μg/mL mitomycin C and grown overnight at 30°C shaking at 150 RPM. Low aeration samples were diluted 1:24 in 1 mL of fresh media contained in individual wells of a 96-well culture box (Axygen Scientfic), and grown to an OD_{600} of   0.5. Protein expression was induced with 1 μg/mL mitomycin C and grown overnight at 30°C shaking at 150 RPM on a microtiter plate shaker.

After 18 hours of induced growth, cultures were serially diluted and plated on LB plates supplemented with 100 μg/mL ampicillin. Plates were incubated overnight at 37°C, and colonies were counted to enable normalized loading and separation of cellular lysate using SDS-PAGE. Detection of expressed protein was done using the Penta-His HRP Conjugate Kit and following the manufacturer's provided protocol (Qiagen).

2.3 Expression and detection of clone set genes

E. coli containing expression plasmids for each gene of interest were grown overnight in 1 mL of LB broth in 96-well, round bottom deep-well culture boxes shaking at 900 RPM. Overnight cultures were diluted and induced as described above. The following day, cultures were centrifuged for 10 min at $1,278 \times g$ *and* resuspended in 100 μL SDS gel loading buffer (50 mM Tris-Cl (pH 6.8), 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol). The resulting lysate was separated by SDS-PAGE or 2 μL was spotted directly onto nitrocellulose transfer membrane and allowed to dry at 37°C for 30 m. Detection of expressed proteins was achieved using the Penta-His HRP Conjugate Kit and following the manufacturers provided protocol (Qiagen).

2.4 96-well Protein Purification

Proteins were expressed as described above. Three 1 mL cultures of each clone were grown and induced simultaneously. Purification was performed using TALON cobalt resin (Clontech) in a 96-well format for use with a titer-plate centrifuge (Sorvall). After overnight expression and centrifugation, cells were resuspended and lysed using 1 mL of TALON xTractor Buffer (Clontech) supplemented with 6 M urea. Lysates were left shaking at room

temperature for 1 hr following addition of lysis buffer. A TALON HT 96-well plate was centrifuged at $700 \times g$ for 30 s to remove storage ethanol. Beads were washed once in water and then twice in PBS (pH 7.4) supplemented with 6 M urea, and centrifuged after each resuspension at $700 \times g$ for 30 s to remove supernatant. 3 mL of total lysate was used to resuspend the resin and allowed to incubate for 30 min at room temperature, either vortexing or pipetting every five minutes to ensure the resin would not settle out of solution. Flow through was collected by centrifuging at $700 \times g$ for 30 s with a clean 96-well plate. The resin was washed five times with 200 μL PBS supplemented with 6M urea and 10 mM imidazole, and protein was eluted with two 200μL washes of PBS-urea containing 200 mM imidazole and stored at 4°C. Protein concentration was determined using a Bradford assay (BioRad), and the presence and relative purity of the target protein was determined using the previously described Penta-His HRP kit and a Coomassie stained SDS-PAGE gel, respectively.

2.5 Serum Dot Blots

We examined the seroreactivity of expressed proteins with sera from previous infection studies [39,40], thus eliminating the need for *de novo* infections. Mammalian serum from multiple species was obtained from several collaborators. Rabbit serum (Dr. Johnny Peterson, University of Texas-Galveston) was collected from Dutch-belted rabbits infected intranasally with 1×10^7 CFU of *B. anthracis* Ames spores. The rabbits were passively immunized 24 hours post-infection with a human monoclonal antibody (AVP-21D9) against PA and the serum collected 30 days post-infection and pooled [40]. Mouse serum (Dr. Henry Heine, USAMRIID) was collected from female BALB/c mice infected with 50 LD_{50}s of *B. anthracis* Ames spores *via* aerosolization. At 60 hours post infection, after significant germination and bacteremia had occurred, the mice were treated with 100 mg/kg amoxicillin every 12 hours. Treatment lasted for 21 days, and surviving animals were bled and serum collected 60 days post-infection [39]. Convalescent serum from AVA-vaccinated rhesus macaques that had survived inhalational anthrax was provided by Dr. Conrad P. Quinn at the Centers for Disease Control in Atlanta, GA. Rhesus macaques were immunized with three intramuscular doses (weeks 0, 4, and 26) of AVA and survived aerosol challenge with 200-400 LD_{50} equivalents of *B. anthracis* Ames strain at week 52. Sera were harvested on days 14 and 30 post-challenge. Finally, human serum from AVA-vaccinated individuals was provided by Dr. Wendy Keitel (Baylor College of Medicine). These subjects were vaccinated using the original AVA schedule, which used 6 subcutaneous injections instead of 5 (0, 2, and 4 weeks, followed by injections at 6, 12, and 18 months, with yearly boosters thereafter.) Three samples were collected from five subjects and assayed individually. Samples were collected pre-injection, 1 month after the first injection, and 1 month after the second injection.

To screen for seroreactivity, 2 μL of expressed and purified proteins were spotted directly onto nitrocellulose transfer membrane and allowed to dry for 30 min at 37°C. Blots were then rinsed twice for 10 min with TBS and blocked overnight at 4°C with TBS containing 2% milk and 0.1% Tween-20, or PBS containing 2% milk for the human serum blots. Blots were rinsed twice with TBS with 0.1% Tween (TBST) and once with TBS before exposure to primary serum. The serum was diluted 1:20,000 in blocking buffer and allowed to incubate for 1 hr at room temperature. Blots were washed twice with TBST, once with TBS, and then exposed to secondary antibody. The secondary rabbit antibody was a goat anti-rabbit IgG, IgM, IgA conjugated to HRP (Open Biosystems). The secondary mouse antibody was a rabbit anti-mouse IgG, IgM antibody conjugated to HRP (Thermo Scientific). Secondary macaque antibody was a goat anti-monkey IgG, IgM, IgA antibody conjugated to HRP (Open Biosystems). Secondary human antibodies were goat anti-human IgG, IgM, IgA antibodies conjugated to HRP (Open Biosystems). Secondary antibodies were diluted

1:7,500 in blocking buffer and allowed to incubate on the membrane for 1 hr at room temperature. Membranes were washed twice with TBST and once with TBS and developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce).

3. Results

3.1 Cloning of *B. anthracis* virulence plasmid clone set

Downstream genomics and proteomics projects often involve libraries with large numbers of clones, and high-throughput expression of these libraries can require hundreds of cultures and growth in relatively small volumes. Growing such cultures in 96-well boxes creates low-oxygen conditions that are generally regarded as sub-optimal for standard protein expression protocols. To address this, we adapted a system used by Eraso and Weinstock [37], which uses the *cea-kil* operon to drive protein expression under SOS-inducing, oxygen limiting conditions, and combined this promoter with a ligation-independent cloning system (Figure 1, 2). The SOS-inducible *cea* promoter was isolated from its original plasmid backbone, pGE124 [37]. The cloning plasmid, pHG7, was created as described in Materials and Methods and enabled the use of a consistent primer design to amplify and clone every pXO1 and pXO2 gene, each with a common complimentary region designed to anneal to the plasmid. The terminal expression vector added 10x-His and T7 epitope tags to each protein product (Figure 1).

3.2 Expression of pXO1/pXO2 clone set genes

E. coli clones harboring individual *B. anthracis* virulence-plasmid genes under control of the *cea* promoter were first used to demonstrate that our high throughput system facilitated increased protein expression under the low-oxygen conditions typical of 96-well box cultures. Protein expression was induced under both high- and low-aeration conditions, and these samples were run on a Western blot and visualized using an anti-His antibody. Figure 3 illustrates four clones that showed increased expression under these low-aeration conditions. Of the subset of clones tested in this fashion (n=20), approximately 54% were characterized by improved protein expression under low-aeration conditions.

We then characterized protein expression of the full set of clones after induction with mitomycin C under low aeration conditions. Lysates were spotted directly onto nitrocellulose membranes and visualized by Western blot (Figure 4). Because a very large number of clones were being evaluated during each screen, sample loading was not normalized. Once protein expression was verified using this dot blot technique, size was verified using SDS-PAGE and Western blot. Of the 207 expression clones tested, 130 of them showed positive expression, and of those 130 proteins, 117 of them expressed at or near the predicted size (Table 3). The 13 proteins that expressed below their predicted size were nonetheless included in the serum screens described below, as these truncated products may still contain antibody-binding epitopes.

Analysis of the genes that expressed well in our system indicated that 57 of the 130 (44%) protein products were previously annotated as hypothetical proteins, and upon our evaluation, we found that they possess no significant homology to any characterized proteins. The remaining proteins possess a wide range of functions. 24 are associated with nucleic acid interaction, including DNA binding, transcriptional regulation, and recombination. Of these 24, 11 were specifically related to transposons and their associated integrases and transposases. Another ten proteins were associated with external structures in *B. anthracis,* including spore, capsule, and Slayer associated proteins and regulators (Supplementary Table 1).

We also examined the genes that expressed minimally, or not at all, in our system. Among those, almost 62% are predicted hypothetical proteins. Specifically, 15 of the 18 poorly expressed genes on the pXO2 plasmid are annotated as hypothetical. Among those that had been assigned a function, five were associated with transposons and four were associated with DNA binding (Supplementary Table 1).

3.3 High-throughput protein purification and mammalian serum screening

Our initial attempts to characterize seroreactivity in our library used crude bacterial lysate after mitomycin C induction. These studies were inconclusive, because a high level of background signal made the dot blots unreadable. To address this issue, we purified the expressed proteins using the TALON 96-well purification system (Clontech, Mountain View, CA), which binds histidine using a cobalt metal ion in a high-throughput format. Two examples of purified protein using this strategy, pXOl-122 and pXOl-65, are shown in Figure 5. After purification, all samples were subjected to Bradford assays, which indicated that each clone produced an average total yield between 20 and 50 μg of protein from 3 mL of culture.

To identify which purified proteins were seroreactive, serum from three mammalian infection models (Rhesus macaque, Dutch-belted rabbits, and BALB/c mice) was used to probe the purified proteins. Upon visualization of the serum blots, signals significantly higher than background were selected as positive signals. Three screens using rabbit serum, two screens using mouse serum, and two screens using macaque serum were performed for each protein. Our screens identified a group of 17 proteins that reacted with sera from at least two of three infection models. Of these, 10 reacted with sera from all three infection models, including six hypothetical proteins, an S-layer amidase, and an ABC transporter. PA served as a positive control in these experiments. The dot blot results from these 10 proteins can be found in Figure 6, and a collective list of proteins identified in individual infection systems can be found in Table 4. Serum from uninfected, negative control rabbits showed no reactivity with any of the proteins that were screened, indicating that the proteins detected using the rabbit serum resulted from an antibody response during the initial *B. anthracis* infection.

Finally, the purified proteins were used to determine whether any are detected by the humoral response following the AVA vaccination. It is generally accepted that PA is the primary immunogenic component of this vaccine, but it is possible other targets may contribute to the immune response. To test our purified proteins against the human antibody response, serum collected from individuals vaccinated with the AVA vaccine was used in a serum dot blot. Though we were able to see a clear, increasing response to PA throughout the vaccination schedule (Figure 7), there were no other signals common between the infection models and vaccination model.

4. Discussion

The current standard for anthrax vaccination, the AVA vaccine, has come under increasing scrutiny since the terrorist attacks in the United States in 2001. While effective, the licensed vaccine schedule is expensive and cumbersome. Furthermore, concerns regarding the safety and lot to lot variability of AVA have incited research into alternatives to and improvements of this standard.

Recombinant protein vaccines have the potential to be used to protect against anthrax, and considering that the AVA vaccine is composed primarily of PA, a recombinant vaccine combining PA with another protein (or collection of proteins) may increase protection and address concerns about safety and reproducibility in a more tightly controlled manner

[27,42]. There has been increasing focus on accurate characterization of the antigen profile of *B. anthracis,* and the findings from some of these studies are summarized in Table 1 and in Chitlaru et al. [35]. However, a majority of the studies characterizing these proteins have largely focused on chromosomal proteins or a small subset of virulence plasmid proteins. Our novel approach was designed to target the entirety of the virulence plasmid-encoded proteins to determine whether the virulence plasmids contain multiple, previously uncharacterized immunoreactive targets. To carry out this investigation, we adapted a ligation-independent cloning technique using the ColE1 promoter to allow for use in low-aeration conditions and of the SOS response to mitomycin C to drive expression of recombinant proteins [37]. ColE1-driven expression of these proteins was improved under low aeration conditions, and approximately 57% of these clones expressed as full-length proteins detected by Western blot at the predicted size. This cloning technique was chosen because it enabled cloning of a large number of *B. anthracis* genes with a single primer design without the need for relatively expensive recombinases and produced more efficient expression under the unique low-aeration conditions experienced during growth in 96-well boxes.

One strength of this system lies in its ability to be used in any project aiming to create and express a large clone set. With the exception of primer design, there should be little need to vary the protocol described here for future projects, and the SOS expression mechanism is optimized for oxygen conditions common in 96-well culture boxes. In addition, the ability to purify recombinant proteins from this high-throughput expression system contributes further to its strength. It is also noteworthy that these studies describe expression of Gram-positive *B. anthracis* genes in a Gram-negative *E. coli* strain, and as such these conditions are likely suboptimal for expression of *B. anthracis* genes. Attempts to express proteins from a Gram-negative system may result in greater success than was observed here.

To this point, some observations were made that could speak to general expression of heterologous proteins from *B. anthracis* in *E. coli.* While 44% of the proteins that expressed well have been annotated as hypothetical proteins, 18% of those that did have an annotated function were associated with DNA binding. Of the DNA binding proteins, 45% were associated with transposase biology. The high frequency of transposase-related proteins is not surprising. An increased understanding of transposes in general has allowed new genome sequencing projects to accurately identify these genes in their respective organisms. In addition, transposons are often associated with plasmids, providing their hosts with the ability to modify chromosomal genes and sustain a limited degree of variability that may ultimately benefit their overall fitness. An unexpected result was that we observed reasonable expression of proteins associated with external structures in *B. anthracis,* including components of the S-layer, spore, and capsule. Considering that these structures are unique to Gram-positive bacteria, faulty expression might have been expected in our Gram-negative system.

Screening expressed proteins with serum from multiple infection models identified 17 proteins that were immunogenic in at least two of our animal infection models. Importantly, 15 of these had not been previously identified in other studies, and eight of the 15 were immunoreactive in all three models. Further studies characterizing these candidates will reveal which, if any, may provide protection in a recombinant protein-based vaccine. Many of the immunoreactive proteins are annotated as hypothetical proteins. However, pXO2-42, found to be reactive in immunoreactivity screens here and elsewhere [22,23], is an S-layer amidase predicted to localize to the external cell surface. This suggests that pXO2-42 is readily accessible to the humoral immune response and therefore should be prioritized in future protection studies.

In addition, all previously reported immunogenic proteins that we expressed in the system described here were also found to be immunoreactive, suggesting that this reactivity is not due to any *B. anthracis*-specific post-translational modifications. This information may prove useful if the need arises to produce any of these proteins in heterologous systems for vaccine trials.

While the AVA vaccine screen did not identify any targets other than the expected antibody interaction with PA, it provided further evidence that a multi-component subunit vaccine may be optimal to obtain maximum protection. If antibodies are raised against a greater number of targets during a natural infection, it would follow that there are a number of additional subunit candidates which may enhance protection compared to using PA only. Further studies will reveal which of the candidates described here may afford protection and could be combined with PA to create an improved protein-based recombinant vaccine against anthrax.

5. Conclusions

For cloning and expression of large numbers of genes, the ligation-independent cloning method described here was shown to be efficient and amenable for high-throughput DNA manipulations, protein expression and protein purification. Application of this technique to express the genes encoded on the *B. anthracis* pXO1 and pXO2 virulence plasmids led to the subsequent identification of 10 immunoreactive and/or antigenic proteins that could enhance the protective capacity of the current AVA vaccine or contribute to the design of a completely new anthrax vaccine

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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We created a high-throughput expression clone set for *B. anthracis* pXO1 and pXO2 plasmids. > We adapted ligation-independent cloning technique for expression in low-oxygen conditions. > Expression totals and quality was determined. > Animal serum was used to identify 8 previously unidentified immunogenic proteins.
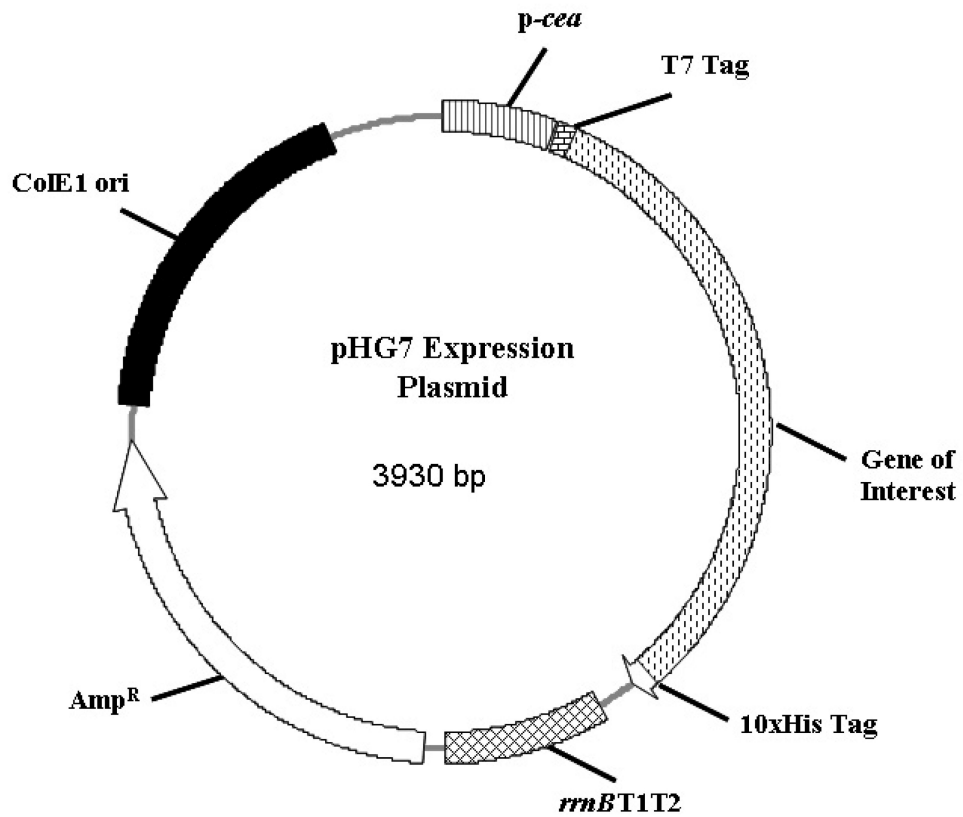
Figure 1.
Plasmid pHG7, engineered for expression of proteins under microaerobic conditions. Expression is driven by the SOS response, which induces the *cea* promoter. Expression produces a protein product with an N-terminal T7 tag and a C-terminal His tag.
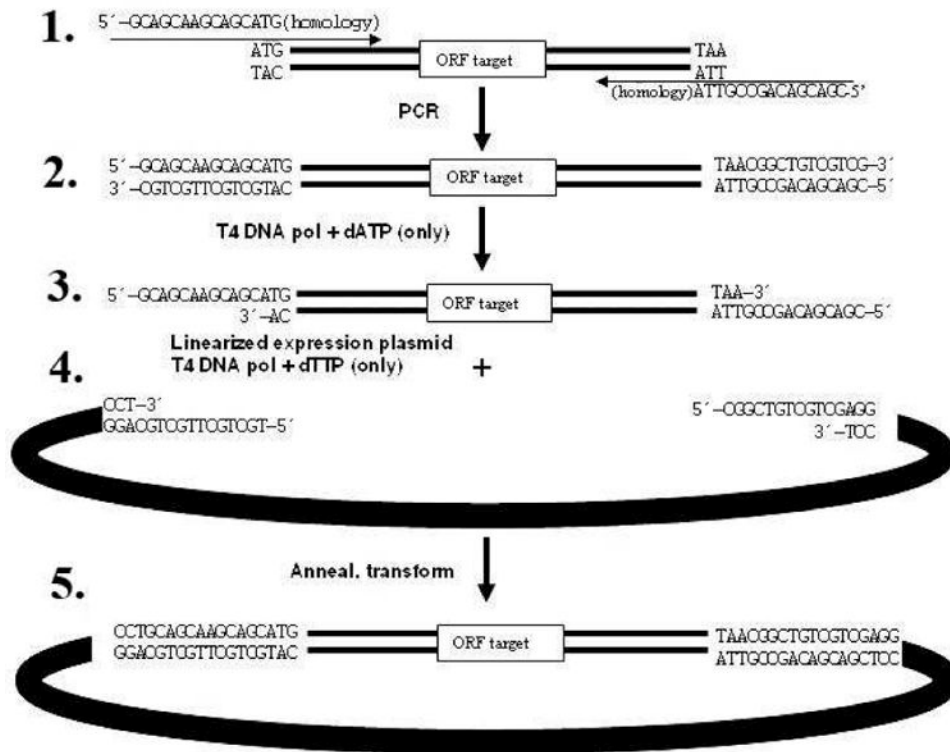
Figure 2.
Ligation-independent cloning using pHG7: (1) An ORF target is amplified using primers with extensions homologous to the DNA ends generated by linearization of the expression vector. After amplification (2), the PCR product is treated with T4 DNA polymerase in the presence of one dNTP (dATP in this example). (3) 3′-5′ exonuclease activity results in a 5′ overhang and a terminal dATP on the opposite strand. This product can be annealed (4) to similarly treated plasmid DNA (with dTTP the only included dNTP) to generate a recombinant plasmid upon transformation (5).
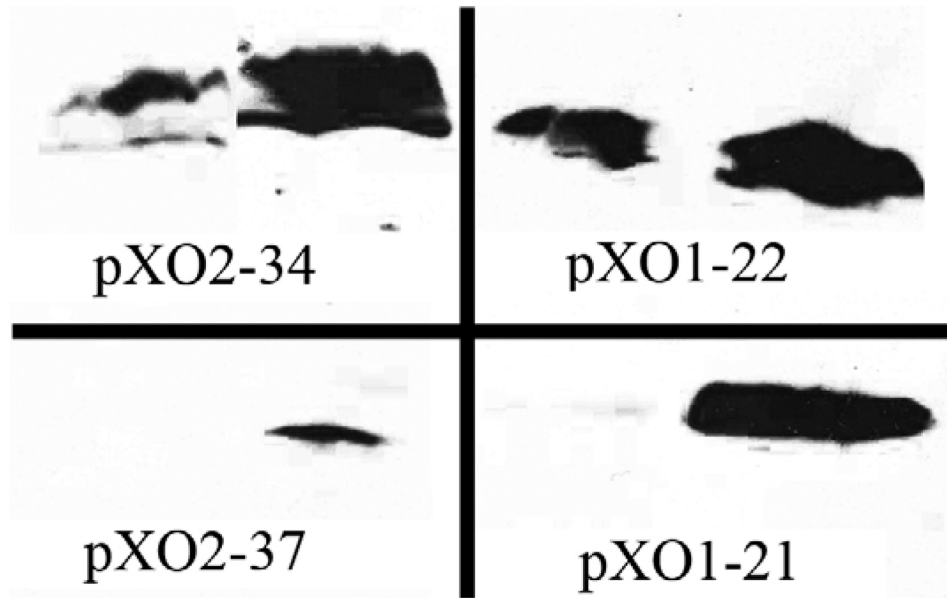
Figure 3.
Protein expression under high- and low-aeration conditions. Cultures of each clone were grown overnight and diluted into either a 250 mL flask (high aeration, left sample) or a single well in a 96-well box (low aeration, right sample). Both cultures were induced, grown overnight, and run on a Western Blot, where loading was normalized by serial dilutions and plating. All proteins were visualized at the expected size.
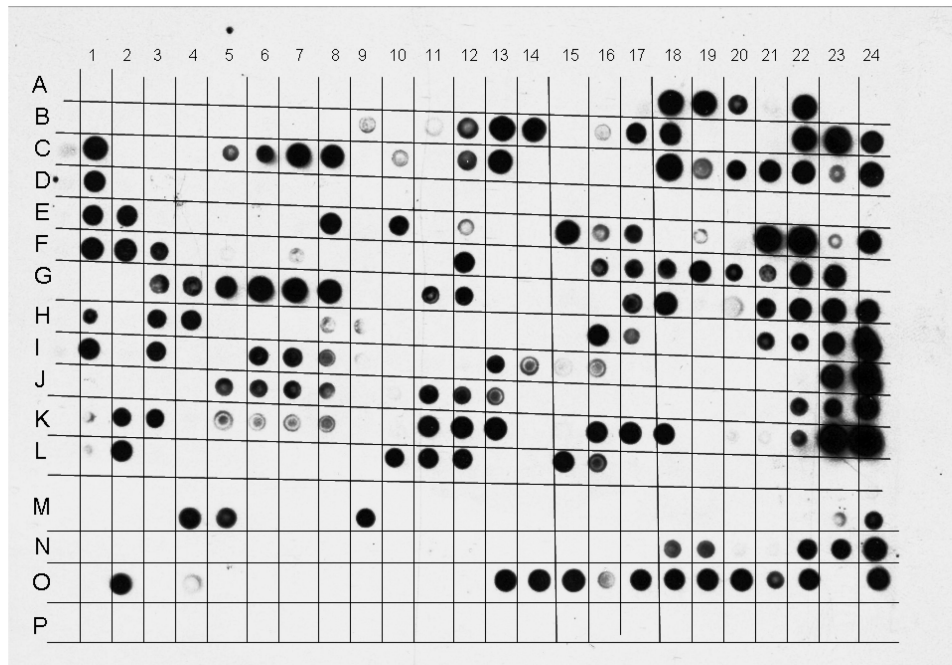
Figure 4.
Anti-His dot blot showing expression of four individual 96-well boxes of expressed clones.
The first two rows of a given 96-well box are spotted here in row "A," the next two in row
"B", and so on through row "D," where the last two rows of a given box are included. Cells
were induced with mitomycin C, lysed, and 2 μL of lysate spotted for visualization. Array
runs 16 rows by 24 columns. An example of positive expression is visible at CI3, which
corresponds to the gene pX02-5 (GI: 6470156). An example of low but acceptable
expression is seen in C19, which corresponds to gene pX02-15 (GI: 6470166.)
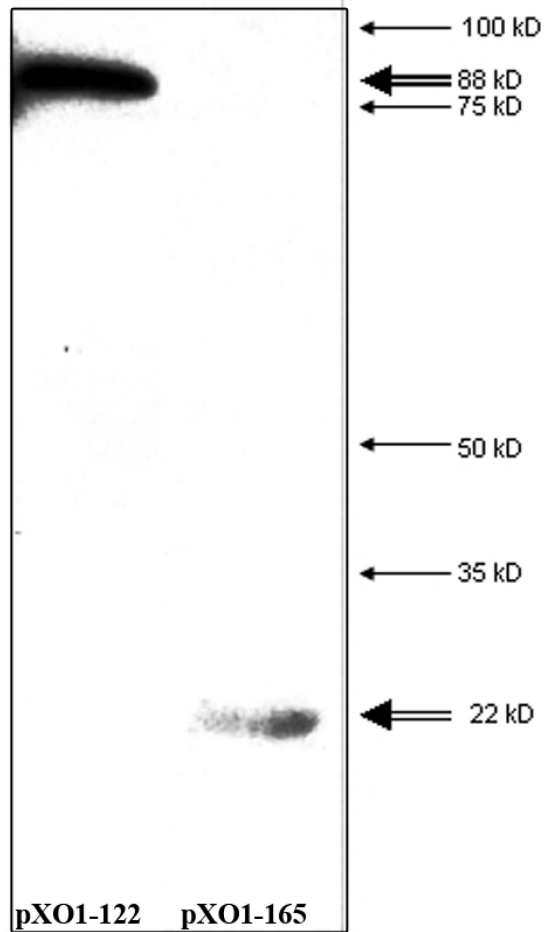
Figure 5.
Western blots of two purified proteins using the 96-Well TALON method. The Western blot was performed with a Penta-HIS kit (Qiagen). The left sample is pXOl-122, which has a predicted size of approximately 88 kD, and the right sample is pXOl-65, which has a predicted size of 22 kD. These predicted sizes are indicated by the double arrow. The single arrows indicate the location of four molecular weight bands from the Precision Plus Protein Standards protein ladder (BioRad).
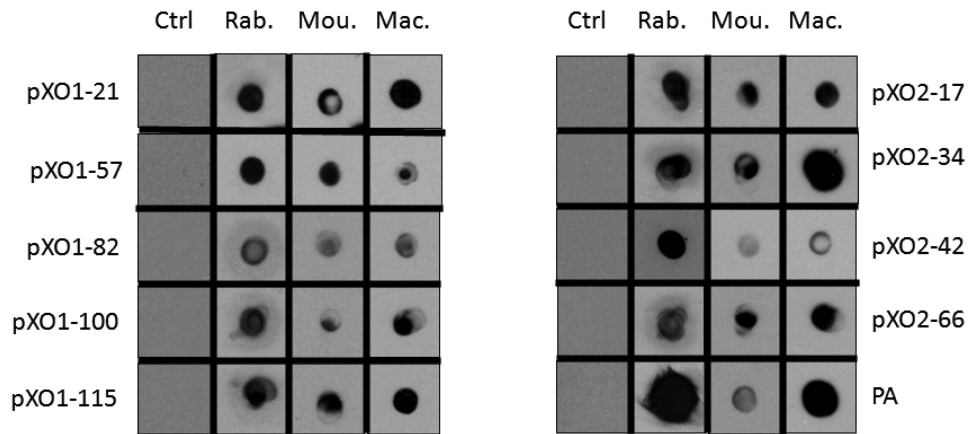
Figure 6.
Serum blot results from pre-infected rabbits (left, "Ctrl"), infected rabbits (Rab.), infected mice (Mou.) and infected macaques (Mac.). In each case, primary antibody was visualized by using a species-specific, HRP-conjugated secondary antibody as described in Materials and Methods.
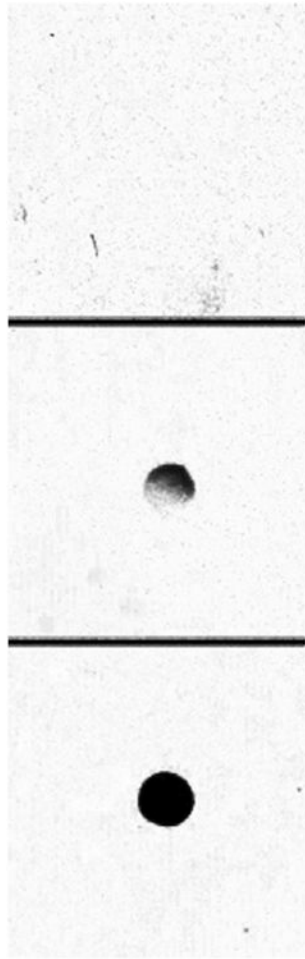
Figure 7.
Recombinant PA visualized using serum from AVA-vaccinated human subjects. Top panel shows pre-vaccination control serum. Middle panel shows binding of serum after first AVA injection, and bottom panel shows binding of serum after final AVA injection.

Table 1

Virulence-plasmid associated seroreactive proteins that have been identified since 2002. Mammalian systems include mica [M], rabbits [R]. macaques [Q], guinea pigs, [G], and humans [H]. "*" denotes a protein identified in multiple studies

Study	Locus Tag	Protein	Model
This study	pXOl-21	Reductase	MRQ
	pXOl-57	Hypothetical protein	MRQ
	pXOl-52	Hypothetical protein	MRQ
	pXOl-100	Hypothetical protein	MRQ
	pXOl-110*	Protective antigen (PA)	MRQ
	pXOl-115	Hypothetical protein	MRQ
	pX02-17	Hypothetical protein	MRQ
	pX02-34	Hypothetical protein	MRQ
	pX02-42*	S-layer amidase	MRQ
	pXO2-66	ABC transporter	MRQ
Ariel 2002	pX01-54*	S-Layer protein	G
	pXOl-90*	S-Layer, DNA interaction	GR
	pXOl-110*	Protective antigen (PA)	R
	pXOl-130	SBP-ABC [Zn] (YodA)	R
Gat 2006	pXO-54*	S-Domain containing protein	G
	pXO1-90*	S-Layer, DNA interaction	R
	pXOl-107*	Lethal factor (LF)	R
	pXOl-110*	Protective antigen (PA)	R
	pXOl-122*	Edema factor (EF)	R
	pXOl-130*	SBP-ABC [Zn] (YodA)	R
	pXO2-08*	NLP/P60 lipoprotein family	G
	pX02-42*	Amidase	R
Chitlaru 2007	pXOl-54*	S-Domain containing protein	R
	pXOl-90*	S-Layer, DNA interaction	R
	pXOl-107*	Lethal factor (LF)	R
	pXOl-110*	Protective antigen (PA)	R
	pXOl-122*	Edema factor (EF)	R
	pXOl-130*	SBP-ABC [Zn] (YodA)	R
Walz 2007	pXOl-110*	Protective antigen (PA)	H
Rollins 2003	pXOl-110*	Protective antigen (PA)	Q
	pXO2-08*	NLP/P60 lipoprotein family	Q
	pX02-42*	Amidase	Q
	BXB0048*	Hypothetical protein	Q

Table 2

Primers and Plasmids Table

HG1	5 -GGGGTTTTAATATTGAGAACGGTAATGTTTGTGCTGG-3
HG2	5 -GGGGTTTTGGATCCTATAAAATCCTCTTTGACTTTT-3
HG3	5 -GGGGTTTTCATATGGAGAACGGTAATGTTTGTGCTGG-3
HG4	5 -GGGGTTTTGAATTCTATAAAATCCTCTTTGACTTTT-3
HG50	5 -GGGGTTTTGAATTCTCTAGAGGATCCGTCGACCTGCAGATGAAATCTAACAATGCGCTCATCG-3
HG52	5 -GGGGTTTTAAGCTTTTATTAGTGATGGTGATGGTGATGGTGATGGTGATGCCCGGGGGTCGAGGTGGCCCGGCTCC ATGC-3
HG53	5 -GGGTTTGCATATGGAGAACGGTAATGTTTGTGC-3
HG54	5 -GGGTTTGGAAGCGGAAGAGCGGGTTATTGTCTCATGAGCGG-3
HG55	5 -GGGTTTTGAATTCATGGCTAGCATGACTGGTGGACAGCAAATGGGAGGAGCGGCCGCGAAATCTAACAATGCGCT CATCGTCATCC-3
HG56	5 -GGGGTTTTAAGCTTTTATTAATGGTGATGGTGATGGTGATGGTGATGGTGGGCAGCGCGGCCGCCGGTCGAGGTGG CCCGGCTCCATGCACC-3
pHG2	*cea* promoter amplified from pGE124
pHG4	*tet* gene cloned from pKRP12 and inserted into pHG2
pHG5	*cea* promoter, I0×His tagged *tet* gene, *rrnB*TlT2 fragment of pHG4 cloned into Ndel and Sapl sites of pUCI8
pHG6	N-terminal T7 antigen tag and 5 and 3 LIC sites inserted into EcoRI-HindIII fragment of pHG5
pHG7	pHG4 promoter, T7, His, and LIC cloning site in pUCI8 created by NotI digest of pHG6 and recircularizing the larger fragment (i.e. without *tet)*

Table 3

Data from expression studies of B. anthracis expression clone set.

pXOl	
Total genes on plasmid	145
Total genes tested	129
Expr.	74
%Expr.	57.40%
Expr. + Size	63
% Expr. 4 Size	48.80%

pX02	
Total genes on plasmid	84
Total genes tested	78
Expr.	56
%Expr.	71.80%
Expr. + Size	54
% Expr. + Size	69.20%

Totals	
Total Genes Tested	207
Expr.	130
%Expr.	62.80%
Expr. + Size	117
% Expr. + Size	56.50%

Table 4

List of proteins detected in infection models. [A] indicates detection in mice, [B] rabbits, and [C] in macaques

Seroreactive Proteins	
pXOl-21[ABC]	Reductase
pXOl-57[ABC]	Hypothetical protein
pXOl-82[ABC]	Hypothetical protein
pXOl-l00[ABC]	Hypothetical protein
pXOl-110[ABC]	Protective antigen
pxol-115[ABC]	Hypothetical protein
pXO2-17[ABC]	Hypothetical protein
pX02-34[ABC]	Hypothetical protein
pX02-42[ABC]	S-layer amidase
pXO2-66[ABC]	ABC Transporter
pX01-22[AB]	Hypothetical protein
pXOl-55[BC]	Hypothetical protein
pXO2-33[AC]	Hypothetical protein
pX02-37 [AC]	Hypothetical protein
pX02-52[AC]	Hypothetical protein
pX02-55[AC]	gamma-glutamyltranspeptidase
pX02-59[AC]	Signal peptidase
pXOl-135[B]	Hypothetical protein
pX02-7[C]	Hypothetical protein
pX02-8[B]	Lysozyme-like protein
pX02-26[B]	Hypothetical protein
pX02-64[A]	Capsule synthesis regulator