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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.

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].

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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-γ-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 [23]. 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) [34]. 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.

Table 1
Virulence-plasmid associated seroreactive proteins that have been identified since 2002. Mammalian systems include mice [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	pXO1-21	Reductase	MRQ
	pXO1-57	Hypothetical protein	MRQ
	pXO1-82	Hypothetical protein	MRQ
	pXO1-100	Hypothetical protein	MRQ
	pXO1-110*	Protective antigen (PA)	MRQ
	pXO1-115	Hypothetical protein	MRQ
	pXO2-17	Hypothetical protein	MRQ
	pXO2-34	Hypothetical protein	MRQ
	pXO2-42*	S-layer amidase	MRQ
	pXO2-66	ABC transporter	MRQ
Ariel 2002	pXO1-54*	S-Layer protein	G
	pXO1-90*	S-Layer, DNA interaction	GR
	pXO1-110*	Protective antigen (PA)	R
	pXO1-130	SBP-ABC [Zn] (YodA)	R
Gat 2006	pXO1-54*	S-Domain containing protein	G
	pXO1-90*	S-Layer, DNA interaction	R
	pXO1-107*	Lethal factor (LF)	R
	pXO1-110 *	Protective antigen (PA)	R
	pXO1-122*	Edema factor (EF)	R
	pXO1-130*	SBP-ABC [Zn] (YodA)	R
	pXO2-08*	NLP/P60 lipoprotein family	G
	pXO2-42*	Amidase	R
Chitlaru 2007	pXO1-54*	S-Domain containing protein	R
	pXO1-90*	S-Layer, DNA interaction	R
	pXO1-107*	Lethal factor (LF)	R
	pXO1-110*	Protective antigen (PA)	R
	pXO1-122*	Edema factor (EF)	R
	pXO1-130*	SBP-ABC [Zn] (YodA)	R
Walz 2007	pXO1-110*	Protective antigen (PA)	H
Rollins 2008	pXO1-110*	Protective antigen (PA)	Q
	pXO2-08*	NLP/P60 lipoprotein family	Q
	pXO2-42*	Amidase	Q
	BXB0048*	Hypothetical protein	Q

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 ([7], 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 [35] 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.