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Accessibility
Application of In Vivo Induced Antigen Technology (IVIAT) to Bacillus anthracis

Sean M. Rollins1*, Amanda Peppercorn1, John S. Young1, Melissa Drysdale2,3, Andrea Baresch1, Margaret V. Bikowski1, David A. Ashford4, Conrad P. Quinn5, Martin Handfield6, Jeffrey D. Hillman7, C. Rick Lyons2, Theresa M. Koehler2, Stephen B. Calderwood1,8,9, Edward T. Ryan1,9,10

1 Massachusetts General Hospital, Boston, Massachusetts, United States of America, 2 University of New Mexico Health Science Center, Albuquerque, New Mexico, United States of America, 3 Department of Microbiology and Molecular Genetics, The University of Texas Medical School at Houston, Houston, Texas, United States of America, 4 United States Department of Agriculture (USDA), Animal and Plant Health Inspection Service (APHIS), Sao Paulo, Brazil, 5 Centers for Disease Control and Prevention, Atlanta, Georgia, United States of America, 6 Center for Molecular Microbiology and Department of Oral Biology, College of Dentistry, University of Florida, Gainesville, Florida, United States of America, 7 Oragenics Inc, Alachua, Florida, United States of America, 8 Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts, United States of America, 9 Department of Medicine, Harvard Medical School, Boston, Massachusetts, United States of America, 10 Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, United States of America

Abstract

In vivo induced antigen technology (IVIAT) is an immuno-screening technique that identifies bacterial antigens expressed during infection and not during standard in vitro culturing conditions. We applied IVIAT to Bacillus anthracis and identified PagA, seven members of a N-acetylmuramoyl-L-lalanine amidase autolysin family, three P60 family lipoproteins, two transporters, spore cortex lytic protein SleB, a penicillin binding protein, a putative prophage holin, respiratory nitrate reductase NarG, and three proteins of unknown function. Using quantitative real-time PCR comparing RNA isolated from in vitro cultured B. anthracis to RNA isolated from BALB/c mice infected with virulent Ames strain B. anthracis, we confirmed induced expression in vivo for a subset of B. anthracis genes identified by IVIAT, including L-alanine amidases BA3767, BA4073, and amiA (pXO2-42); the bacteriopeptide holin gene BA4074; and pagA (pXO1-110). The exogenous addition of two purified putative autolysins identified by IVIAT, N-acetylmuramoyl-L-alanine amidases BA0485 and BA2446, to vegetative B. anthracis cell suspensions induced a species-specific change in bacterial morphology and reduction in viable bacterial cells. Many of the proteins identified in our screen are predicted to affect peptidoglycan re-modeling, and our results support significant cell wall structural remodeling activity during B. anthracis infection. Identification of L-alanine amidases with B. anthracis specificity may suggest new therapeutic targets.


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* E-mail: srollins1@partners.org

Introduction

In vivo induced antigen technology (IVIAT) is an immuno-screening technique designed to identify immunogenic bacterial genes expressed specifically during infection [1–4]. We hypothesized that applying IVIAT to Bacillus anthracis, the cause of anthrax, could lead to increased understanding of bacterial events during infection, improved diagnostic assays, and novel therapeutic targets. IVIAT utilizes convalescent sera from patients or animals infected with a pathogen of interest [1–4]. Convalescent sera are pooled from several individuals and adsorbed against the cognate pathogen grown under standard laboratory culture conditions. Extensive adsorptions are performed to remove antibodies that bind bacterial antigens expressed in vitro, while retaining antibodies that recognize bacterial antigens specifically expressed during in vivo growth. Adsorbed serum is then used to probe an Escherichia coli-based inducible protein expression library of the pathogen. IVIAT thus provides a screen to identify proteins that generate a humoral immune response, and are displayed specifically during infection.

Thus far, IVIAT has been applied to several pathogenic bacteria including Vibrio cholerae, Vibrio vulnificus, E. coli O157:H7, Salmonella enterica serovar Typhi, Streptococcus pneumoniae (Group A), and Mycobacterium tuberculosis, among others [2,3,5–9]. In these applications, IVIAT has identified pathogen genes expressed uniquely in vivo, including antigens previously established to be expressed in vivo, and newly recognized virulence factors [2,3,5–9]. Identification of up-regulated virulence genes by IVIAT has been confirmed in complementary studies, including gene microarray analysis, quantitative real time PCR (RT-PCR), signature tagged mutagenesis, and recombinase in vivo expressed technologies (RIVET), among others [9–11].

Certain in vitro conditions have been identified that induce expression of B. anthracis genes involved in pathogenesis during anthrax, including expression of toxins and capsule. For instance, growth of B. anthracis in Nutrient Broth Yeast extract medium...
Many genes encoding proteins involved in encapsulation are on the large virulence plasmid pXO1 (182 kb; 143 ORFs), while genes encoding toxin proteins PA (encoded by pagA) and lethal factor (LF) are on pXO2 (96 kb; 85 ORFs). The presence of capsule impedes opsonophagocytosis and clearance of B. anthracis, which is comprised of poly-D-glutamic acid. The presence of capsule also correlates with the serotype of B. anthracis (selected to mimic the physiology of blood) is associated with increased expression of protective antigen (PA) and capsule compared to that observed during growth in standard laboratory media such as Brain Heart Infusion (BHI) in the presence of air (0.03% CO2) at 37°C [12]. Although PA itself is non-toxic, PA binds specific host cell receptors and forms a pre-pore, which binds either edema factor (EF) or lethal factor (LF) to create edema toxin (ET) and lethal toxin (LT), respectively [13]. Anti-PA immune responses correlate with protection from anthrax, and PA is a primary immunogen in existing anthrax vaccines [14]. Full virulence of B. anthracis also correlates with presence of B. anthracis capsule, which is comprised of poly-D-glutamic acid. The presence of capsule impedes opsonophagocytosis and clearance of B. anthracis organisms from infected hosts [15]. Un-encapsulated strains of B. anthracis are markedly attenuated in both humans and animals, despite their ability to express fully functional LT and ET toxins [16]. Beneath the capsule is a proteinaceous S-layer, composed of two paracrystalline proteins (extractable antigen 1 [EA1] and surface array protein [SAP]) that cover the bacterial cell surface [16]. Beneath the S-layer is a peptidoglycan layer comprising the cell wall.

B. anthracis contains two large virulence plasmids: genes encoding toxin proteins PA (pagA), LF (lef), and EF (cya) are on the large virulence plasmid pXO1 (182 kb; 143 ORFs), while genes encoding proteins involved in encapsulation are on the large virulence plasmid, pXO2 (96 kb; 85 ORFs). Many B. anthracis genes on pXO1, pXO2, and the single large chromosome have not been characterized, and it is possible that additional factors induced during infection and not under typical in vitro culture conditions contribute to B. anthracis pathogenesis. Identification of such gene products could improve our understanding of host-pathogen interactions during anthrax, and could assist further development of appropriate therapeutic, diagnostic, or preventative measures for anthrax.

To identify antigenic B. anthracis proteins displayed specifically during B. anthracis infection, we applied IVIAT using convalescent sera from partially immunized macaques surviving aerosol challenge with wild type B. anthracis Ames spores, quantified expression of identified genes in a mouse model of virulent B. anthracis Ames infection, and performed preliminary functional analysis on a subset of identified B. anthracis gene products.

### Results and Discussion

#### Identification of B. anthracis proteins expressed in vivo by IVIAT

Using IVIAT, we immuno-screened an approximately 125,000 clone inducible B. anthracis expression library in E. coli BL21(DE3) (17,000 pXO1 clones, 7,500 pXO2 clones, and 100,000 chromosomal clones). We identified 60 immuno-reactive clones, representing 25 distinct B. anthracis genes or gene fragments. Of these, we constructed full length constructs and confirmed immuno-reactivity of ten (Table 1). Significant immuno-reactivity

<table>
<thead>
<tr>
<th>Functional category</th>
<th>ORF</th>
<th>Gene product-description, function (reference)</th>
<th>Predicted cellular location</th>
<th>Gene encoded by</th>
<th># of clones identified by primary screening/ # of independent clones identified by primary screening</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virulence</td>
<td>pXO1-110 (pagA)</td>
<td>Protective antigen</td>
<td>Extracellular</td>
<td>pXO1</td>
<td>3/3</td>
<td>Known virulence factor [45,46]</td>
</tr>
<tr>
<td>Transport</td>
<td>BA0314</td>
<td>Substrate-binding protein of an ABC transporter</td>
<td>Membrane associated</td>
<td>Chromosome</td>
<td>1/1</td>
<td>Predicted ABC metal ion transporter; contains lipoprotein domain</td>
</tr>
<tr>
<td>Transport</td>
<td>BA0669 (rbsB)</td>
<td>Ribose binding protein of a ribose ABC transporter</td>
<td>Membrane associated</td>
<td>Chromosome</td>
<td>1/1</td>
<td>Contains membrane-associated signal peptide sequence</td>
</tr>
<tr>
<td>Metabolism</td>
<td>BA2125 (norG)</td>
<td>Respiratory nitrate reductase, alpha subunit</td>
<td>Unknown</td>
<td>Chromosome</td>
<td>3/1</td>
<td>May assist organism to persist in anaerobic conditions [47]</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA2748 (sleB)</td>
<td>Spore cortex lytic protein</td>
<td>Unknown</td>
<td>Chromosome</td>
<td>1/1</td>
<td>See text</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA4073 (plyL)</td>
<td>N-acetylmuramoyl-L-alanine amidase</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>8/4</td>
<td>See text [17]</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA4606 (pdpI)</td>
<td>Penicillin binding protein</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>1/1</td>
<td>See text</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>pXO2-08</td>
<td>NLP/P60 lipoprotein family</td>
<td>Extracellular, cell-associated</td>
<td>pXO2</td>
<td>28/11</td>
<td>See text</td>
</tr>
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<td>Hypothetical</td>
<td>BX80048</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
<td>pXO2</td>
<td>1/1</td>
<td>See text</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>BA0598</td>
<td>Hypothetical protein</td>
<td>Unknown</td>
<td>Chromosome</td>
<td>1/1</td>
<td>See text</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>BA4074</td>
<td>Prophage holin</td>
<td>Membrane associated</td>
<td>Chromosome</td>
<td>8/4</td>
<td>See text</td>
</tr>
<tr>
<td>Hypothetical</td>
<td>BA5474</td>
<td>Degenerative enterotoxin</td>
<td>Unknown</td>
<td>Chromosome</td>
<td>4/1</td>
<td>See text</td>
</tr>
</tbody>
</table>

(NBY) in the presence of 5–20% CO2 and sodium bicarbonate at 37°C (selected to mimic the physiology of blood) is associated with increased expression of protective antigen (PA) and capsule compared to that observed during growth in standard laboratory media such as Brain Heart Infusion (BHI) in the presence of air (0.03% CO2) at 37°C [12]. Although PA itself is non-toxic, PA binds specific host cell receptors and forms a pre-pore, which binds either edema factor (EF) or lethal factor (LF) to create edema toxin (ET) and lethal toxin (LT), respectively [13]. Anti-PA immune responses correlate with protection from anthrax, and PA is a primary immunogen in existing anthrax vaccines [14]. Full virulence of B. anthracis also correlates with presence of B. anthracis capsule, which is comprised of poly-D-glutamic acid. The presence of capsule impedes opsonophagocytosis and clearance of B. anthracis organisms from infected hosts [15]. Un-encapsulated strains of B. anthracis are markedly attenuated in both humans and animals, despite their ability to express fully functional LT and ET toxins. Beneath the capsule is a proteinaceous S-layer, composed of two paracrystalline proteins (extractable antigen 1 [EA1] and surface array protein [SAP]) that cover the bacterial cell surface [16]. Beneath the S-layer is a peptidoglycan layer comprising the cell wall.

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of clones containing in-frame protein-coding fragments of BXB0048 and BA2125 and lacking gene fragments of other Bacillus anthracis genes was detected [these protein coding regions are not similar to any alternative B. anthracis proteins]; however, immuno-reactivity could not be confirmed in clones containing the complete ORFs of these gene fragments, possibly suggesting insufficient expression or display of full-length products in E. coli. Since a number of identified seroreactive ORF products were predicted to be cell wall active amidases or hydrolases, to evaluate whether immuno-reactivity was unique to a given IVIAT-identified ORF, we also evaluated immuno-reactivity of paralogs of IVIAT-identified genes BA4073 (a predicted amidase) and pXO2-08 (a possible hydrolase and our most frequently identified ORF-product), and identified eight additional gene products with immuno-reactivity (Table 2).

### Table 2. Immunoreactive paralogs of Bacillus anthracis gene products identified by IVIAT.

<table>
<thead>
<tr>
<th>Functional category</th>
<th>ORF</th>
<th>Gene product-description, function (reference)</th>
<th>Predicted cellular location</th>
<th>Gene encoded by</th>
<th>Paralog of IVIAT-identified gene product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA4085</td>
<td>N-acetylmuramyl-L-alanine amidase</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>BA4073</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA2446</td>
<td>N-acetylmuramoyl-L-alanine amidase</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>BA4073</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA2805 (plyP)</td>
<td>N-acetylmuramoyl-L-alanine amidase; glycosyl hydrolase [48]</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>BA4073</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA3737</td>
<td>N-acetylmuramoyl-L-alanine amidase</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>BA4073</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>BA3767</td>
<td>N-acetylmuramoyl-L-alanine amidase</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>BA4073</td>
</tr>
<tr>
<td>Peptidoglycan architecture</td>
<td>pXO2-02 (amiA)</td>
<td>N-acetylmuramoyl-L-alanine amidase [49]</td>
<td>Extracellular, cell-associated</td>
<td>pXO2</td>
<td>BA2446, BA3737</td>
</tr>
<tr>
<td>Unknown</td>
<td>BA1952</td>
<td>NLP/P60 lipoprotein family</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>pXO2-08</td>
</tr>
<tr>
<td>Unknown</td>
<td>BAS5427 (lytE)</td>
<td>NLP/P60 lipoprotein family</td>
<td>Extracellular, cell-associated</td>
<td>Chromosome</td>
<td>pXO2-08</td>
</tr>
</tbody>
</table>

For instance, within the NCBI database, the highest similarity protein to L-alanine amidase BA4073 identified by IVIAT is the Bacillus anthracis specific gamma phage lysin, PlyG, with 82% identity over the full length of both proteins, and PlyG has been demonstrated to function as a species-specific B. anthracis lysin [18]. We also identified prophage holin BA4074 by IVIAT. Holins act upon bacterial cytoplasmic membranes and, in conjunction with an endolysin, promote lysis of infected bacteria [19]. Akin to our identification of amidase BA4073 and prophage holin BA4074 by IVIAT, we similarly identified amidase BA3767, which is also in a potential operon arrangement with a prophage holin, BA3768. Using IVIAT, we also identified spore cortex lytic protein (BA2748), a protein with marked similarity to characterized Bacillus cereus and Bacillus subtilis SleB proteins. The latter function as cell wall hydrolases and are required for complete spore-cortex peptidoglycan hydrolysis during germination [20–22]. BA2748 also contains a putative peptidoglycan-binding domain found in a variety of enzymes involved in bacterial cell wall degradation, and expression is induced late during sporulation in B. anthracis [23], although the enzymatic activity in B. subtilis and B. cereus occurs during germination [20,21]. We were also intrigued by our identification of BA4606 that annotates as a penicillin-binding protein. Penicillin-binding proteins are a broad group of membrane-associated macromolecules involved in peptidoglycan biosynthesis, cell wall development, and cell division. BA4606 contains an FtsI cell division protein/penicillin-binding protein 2 domain, and the FtsI penicillin-binding protein family is required for septal peptidoglycan formation during cell division [24]. BA4606 is also 45% identical to B. subtilis hypothetical protein YrrR (also referred to as PBP4h, pbpI) over the full length of both proteins. B. subtilis YrrR is a mother-cell specific penicillin-binding protein expressed during sporulation [25]. The FtsI domain contains a penicillin-binding protein transpeptidase domain, which putatively cross-links peptidoglycan chains to form a rigid cell wall structure.

We also identified four hypothetical proteins. The most frequently identified gene product identified by IVIAT was ORF pXO2-08. pXO2-08 is predicted to be an extracellular protein [26], and contains a P60 family lipoprotein domain. pXO2-08 also has sequence similarity to a murein hydrolase domain from Listeria monocytogenes P60 protein (alternatively referred to as sap-invasion associated protein) [27]. Several proteins containing a P60 domain have been implicated in virulence, including two P60 domain proteins in Mycobacterium marinum involved in invasion and intracellular survival within macrophages [28], and a L. monocytogenes mutant in P60 is severely attenuated, suggesting a potential role in virulence [29]. Although the function of the P60 lipoprotein domain is not completely understood, it is conserved through a variety of bacterial lineages, and some members of this family have been shown to act as murein hydrolases that cleave peptide linkages within peptidoglycan [30]. Immuno-screening of pXO2-08 paralog ORF clones identified two additional Bacillus anthracis serologically reactive paralogs in the P60 lipoprotein family.
BA1952 and BA5427 (byE; putative endopeptidase). BA1952 and BA5427 have been demonstrated to be antigenic secreted proteins, and both contain P60 and S-layer homology (SLH) domains [31-33]. SLH domains are commonly associated with cell surface proteins and non-covalently anchor proteins to the cell wall polysaccharide, suggesting cell surface localization [34]. BA5427 is homologous to B. subtilis byE, and LytE (also referred to as CwlF) has been shown to function as a cell wall lysis that affects bacterial cell separation in B. subtilis [35]. Our application of IVIAT to B. anthracis also identified BA5474, encoding a pseudogene that has sequence similarity to several genes encoding N-acetylmuramoyl-L-alanine amidases and an enterotoxin in B. cereus [36].

RT-PCR quantification of in vivo expression of IVIAT-identified and paralog B. anthracis genes during wild type B. anthracis infection

To further assess in vivo expression of IVIAT-identified ORF products, we measured mRNA levels of antigens identified by IVIAT. Comparing total bacterial RNA recovered from blood, spleen, and lung of mice 6, 12, or 18 hours after inoculation of vegetative B. anthracis Ames, we detected the highest quantity of bacterial RNA in spleen specimens recovered 18 hours after infection. Based on rRNA 16S concentrations, spleen specimens contained bacterial to mouse RNA in ratios ranging from 1:100 to 1:2,000. Comparing RNA recovered from spleen samples to RNA recovered from in vitro cultured organisms grown under the same conditions used to adsorb sera, we detected up-regulation of five B. anthracis IVIAT-identified genes or paralogs/similar proteins in in vivo samples using RT-PCR (Figure 1): pagA; the alanine-amidase genes, BA3767, BA4073, and amk1 (pXO2-42); and the bacteriophage holin gene BA4074. Within our assay, pagA mRNA was present at approximately 54-fold higher levels in in vivo samples than in vitro samples. mRNA of L-alanine amidases BA4073, BA3767, amk1 (pXO2-42), as well as prophage holin BA4074 were also elevated in in vivo samples compared to in vitro samples (33-fold, 24-fold, 16-fold, and 11-fold higher, respectively).

Our identification of PA by IVIAT and our confirmation of up-regulation of pagA mRNA in vivo confirms the validity of our approach since PA is preferentially expressed in vivo and is immunogenic [12,14]. Of note, our inducible B. anthracis library was constructed using B. anthracis strains that did not encode LF or EF, and we would, therefore, not expect our screens to have identified these virulence factors. Our documentation of induced expression of mRNA in vivo of only a subset of genes identified by IVIAT (and not the complete set) is also not unexpected, since mRNA quantity is only one of a number of variables that may alter expression or display of a given protein. Indeed, separate proteomic based analysis has recently demonstrated increased protein expression of two additional B. anthracis gene products that we have identified by IVIAT, lipoproteins BA1952 and BA5427, when B. anthracis are grown in high versus low CO2 concentrations (the former conditions thought to mimic the in vivo environment) [31]. In addition, a number of IVIAT identified antigens (BA0314, BA0485, BA1952, BA2805, BA2748, BA3737, BA427, pXO2-08 and pXO2-42) were also previously identified by Gat et al. using a bioinformatics screening strategy to select B. anthracis ORFs for use in an in vitro transcription/translation assay followed by immunoprecipitation using sera from B. anthracis-infected animals [37]. Identification of these ORF products by both our application of IVIAT to B. anthracis, and by Gat et al.’s screening system, support identified ORF-product immuno-reactivity in vivo.

Species-specific effects of N-acetyl-L-alanine amidases BA0485 and BA2446 on B. anthracis

We were intrigued that a number of the B. anthracis proteins identified in our analysis were predicted to be involved in cell wall peptidoglycan synthesis or remodeling, and that a number were homologous to known lysins. In order to investigate this further, we focused our efforts on the best characterized family of identified proteins, the L-alanine amidases. We were able to purify recombinant versions of two identified amidases, BA0485 and BA2446. Upon addition of exogenous L-alanine amidases BA0485 (buffered at pH 7.0) or BA2446 (buffered at pH 8.7), the OD955 of a cell suspension made from exponential phase organisms fell specifically for B. anthracis, but not for B. cereus, B. subtilis, or E. coli DH15x (Figure 2A). This drop in OD955 corresponded to a 50% reduction in CFU for B. anthracis grown in the presence of BA0485 or BA2446, and this reduction in CFU was not noted in cultures containing BA0485 or BA2446 and B. cereus, B. subtilis, E. coli, or buffer alone (Figure 2B). B. anthracis vegetative bacteria cultured in the presence of exogenous BA0485 or BA2446 also had decreased chain length compared to B. anthracis cultured in buffer alone (BA0485: 1.06 [mean] +/- 0.01 [standard error of mean] versus 2.17 +/- 0.68, p<0.001; BA2446: 1.14 +/- 0.02 versus 2.43 +/- 0.07, p<0.001; Figure 2C-D), as well as a more swollen appearance. The exogenous addition of BA0485 or BA2446 to cultures of B. cereus, B. subtilis, or E. coli had no measurable effect on bacterial morphology, chain length, or viability. Although our exogenous addition of these proteins to in vitro grown cultures may not represent their in vivo location or activity, our data strongly suggest an effect of these proteins on B. anthracis cell wall integrity, a focus of on-going work in our laboratory. We also noted an optimal pH requirement (pH=8.7) for the BA2446 product in vitro, and although similar pH dependence has previously been noted for autolysins specific for B. subtilis [38], the physiological significance of this observation in vivo is currently unclear.

Although our application of IVIAT to B. anthracis has identified a number of genes warranting additional evaluation, our study has a number of limitations. (1) IVIAT only identifies immunoreactive proteins (not carbohydrates or lipids). (2) Our inducible protein library was undoubtedly only partial due to the Sau3A partial digestion used to generate library clones, the effect of codon bias on protein expression, and difficulties expressing all antigens (especially membrane associated proteins) equivalently in E. coli based expression libraries. (3) Immuno-reactivity of IVIAT-identified antigens is a marker of expression of proteins in vivo, and does not predict protective humoral or cellular immune responses. (4) Immuno-reactivity of proteins may also reflect immune responses to related antigens, although we have attempted to identify cross-reactivity in these experiments by also analyzing related proteins. (5) Due to genetic cross-talk between the virulence plasmids and the chromosome of B. anthracis [39], our use in the adsorption protocol of attenuated B. anthracis strains that did not contain the complete genome in a single strain may have misrepresented potential gene expression in vitro. (6) Our use of convalescent phase sera from macaques partially immunized against anthrax and then surviving inhalational challenge with virulent B. anthracis, may not exactly mirror events that occur during natural infection in un-immunized humans or animals, and anti-PA responses in macaques may not correlate with overall anti-B. anthracis immunoreactivity. (7) Our use of the intravenously-challenged mouse model to measure bacterial gene expression in vivo may not exactly reflect events that occur during natural infection (since the mouse model involves neither spore germination nor formation); however, using this model, we were still able...
to detect induction of a number of IVIAT identified genes or paralogs in vivo.

Despite these limitations, our results indicate host antibody responses and increased bacterial production during infection of several proteins, including a subset of seven L-alanine amidases, five proteins containing lipoprotein domains, a prophage holin, spore cortex lytic protein SleB, and a penicillin-binding protein that may be involved in cell wall/peptidoglycan synthesis, degradation, and/or remodeling. Fisher et al. have recently shown that a \textit{B. anthracis} locus (\textit{dltABCD}) involved in cell wall L-alanine esterification is involved in endospore remodeling and is required for full virulence of \textit{B. anthracis} in vivo [40]. Our results further support prominent cell wall structural remodeling activity during anthrax, may suggest new diagnostic targets to detect \textit{B. anthracis} infection, and our identification of predicted L-alanine amidases with \textit{B. anthracis} specificity may suggest new potential alternative therapeutic approaches against this important bacterial pathogen.

**Materials and Methods**

**Bacterial strains and conditions**

\textit{Bacillus} species and strains used in these experiments are described in Table 3. \textit{E. coli} BL21(DE3) and BL21(DE3) Codon...
Plus-RIL strains (Stratagene, La Jolla, CA) were grown in Luria Bertani (LB) media supplemented with appropriate antibiotics. Construction of an inducible B. anthracis genomic DNA expression library

We constructed a BL21(DE3)-based inducible expression library including Sau3A derived 0.5–1.5 kb B. anthracis genomic fragments in pET30abc using DNA from B. anthracis RP42, 9131, and 9131(pXO2) and previously described techniques [3,6,41,42]. Adsorbing convalescent macaque serum to B. anthracis grown in vitro

Healthy rhesus macaques (Macaca mulatta) in the weight range of 2.6 kg to 4.5 kg were quarantined for a minimum of 6 weeks prior to vaccination. Figure 2. B. anthracis species-specific autolysis effects following addition of exogenous putative autolysins N-acetylmuramoyl-L-alanine amidases BA0485 and BA2446: (A) reduction in A_{595} optical density, (B) reduction in colony forming units, and (C, D) morphological changes of B. cereus (C) or B. anthracis (D). B. anthracis (Sterne strain), B. cereus (ATCC 14579), B. subtilis (168), and E. coli (DH5α) vegetative cells were resuspended in 20 mM sodium phosphate buffer containing BA0485 and BA2446 (final concentration 2 μM) or buffer alone (BA0485, pH 7.0; BA2446, pH 8.7). (A) A_{595} readings were recorded every 2 min and reported as a percentage of starting A_{595}. Buffer-only controls were not different for cultures containing B. cereus, B. subtilis, or E. coli. (B) Dilutions of cells were plated prior to addition of BA0485 and BA2446 proteins and then again following 20 min of incubation with buffer alone or buffer containing exogenous BA0485 or BA2446, and CFU are reported as percentage of the starting CFU. (C, D) BA0485, BA2446, or buffer only were added to cell suspensions of B. anthracis, B. cereus, B. subtilis, and E. coli, and incubated for 20 min. Bacterial morphological changes were only evident following protein addition to B. anthracis (D); representative B. cereus samples are shown (C). Gram stains were performed and all images were captured at 1000×.

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to the study start. We obtained convalescent sera from nine macaques that had been immunized with three doses (week 0, 4, 26) of a 1:20 or 1:40 dilution of anthrax vaccine adsorbed (AVA), and survived aerosol challenge with 20–422 LD_{50} equivalents (1.1×10^5–23.2×10^5 CFUs) of B. anthracis Ames strain at week 52. Sera were harvested on days 14 and 30 post-challenge. B. anthracis Ames infection of macaques was confirmed by seroconversion to anti-PA IgG following aerosol exposure. We pooled sera containing the highest anti-PA antibody levels from each surviving macaque. We removed antibodies binding in vitro expressed proteins by six successive direct adsorptions with concentrated whole B. anthracis cells (10^{11} CFUs/ml RP42 and 9131 [pXO2] mixed in equal volumes) grown in vitro in BHI broth in air at 37°C until mid/late log phase [3,6]. We further adsorbed pooled sera with both native and heat-denatured cell lysates of mid/late-log phase cultures of B. anthracis RP42 and B. anthracis 9131(pXO2) (1:1) and E. coli BL21 (DE3), as previously described [3].

Primary screening of the protein expression library, identification of inserts, and prediction of function of antigens identified by IVIAT

For primary screening, we followed a modification of the method of Hang et al [6]. Briefly, we plated aliquots of the protein expression library on LB agar-kanamycin plates and incubated plates for approximately 10 hours at 37°C, and then used nitrocellulose disks to replica plate colonies onto LB agar-kanamycin plates containing 1 mM isopropyl-beta-D-thiogalactoside (IPTG), and incubated plates at 37°C for 3–4 hours. To expose induced antigens, we lysed bacterial colonies by saturating disks with chloroform for ten seconds and air-drying. Following washing and blocking of disks, we incubated membranes with adsorbed sera at a 1:6,000 dilution in PBS-Tween at 4°C overnight with mild agitation, and detected immuno-reactive clones using horse-radish peroxidase-conjugated goat anti-monkey IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD) at a 1:20,000 dilution in PBS-Tween and an ECL chemiluminescence kit with Hyperfilm ECL (Amersham, Pascataway, NJ). We exposed membranes to replica plate colonies onto LB agar-kanamycin plates to determine expression of virulence factors and capsule [43]; we confirmed reactive ORF products with serological immuno-precipitation, removed residual DNA using an Ambion DNAfree kit, and generated cDNAs using an Ambion RTScript kit. Bioinformatically selected paralog and similar protein ORF clones. Specifically, for ORF pXO2-08, we evaluated paralogs BA1952, BA2849, and BA5427; and for ORF BA4073, we evaluated structurally or functionally related amidases including BA0485, BA0872, BA2446, BA2805, BA3737, BA3767, and pXO2-42 (amaA).

Assessment of in vivo expression of IVIAT-identified, paralog and similar B. anthracis genes

To assess in vivo expression of B. anthracis genes identified by IVIAT, we inoculated tail veins of BALB/c mice (Harlan Laboratories, Indianapolis, IN) with 200-LD_{50} of B. anthracis Ames vegetative cells growing in log phase under conditions associated with expression of virulence factors and capsule [43]; we inoculated control mice with PBS. We collected blood, lungs, and spleens of mice sacrificed 6, 12, or 18 hours following inoculation, immediately homogenizing tissues and adding RNAwiz reagent (Ambion; Austin, TX) to recovered samples. We extracted RNA using an Ambion RiboPure-Bacteria kit (Austin, TX), following manufacturer’s specifications. To measure B. anthracis gene expression in vitro, we similarly recovered RNA from Ames strain B. anthracis vegetative cells grown in BHI and air to mid/late log phase. We quantified RNA preparations spectrophotometrically, removed residual DNA using an Ambion DNasefree kit, and generated cDNAs using an Ambion RTScript kit.

We designed primers (Table 4) using Beacon Design (Premier Biosoft; Palo Alto, CA), www.primer3.com, and Blast software. We performed RT-PCR analysis on collected mouse and in vitro grown samples using iQ SYBR Green Supermix reagent (Bio-Rad; Hercules, CA) and a MJ Research Chromo4 thermocycler and MJ Opticon Monitor software version 3.1 (Bio-Rad; Hercules, CA).
After an initial denaturation at 95 °C for 3 min, the RT-PCR cycle was as follows: denaturation at 94 °C for 30 sec, extension at 58 °C for 30 sec, extension at 72 °C for 1 min, followed by a plate read. The cycle was repeated for a total of 40 cycles. C(t) values were set at 1 min, followed by a plate read.

The cycle was repeated for a total of 40 cycles. C(t) values were set at 1 min, followed by a plate read.

**Table 4. Primers for RT-PCR quantification of cDNA isolated from Ames strain B. anthracis recovered from infected mice or from in vitro grown B. anthracis.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>165</td>
<td>TAGGTTGCAAGCGGTATAC</td>
<td>GGTTGCTTCTCATATCTTAC</td>
</tr>
<tr>
<td>BA0314</td>
<td>CGAGAAATACCTCTGGTTCGGAT</td>
<td>GGTTACCGTAGGAGAGAG</td>
</tr>
<tr>
<td>BA0669</td>
<td>ATGCAATGACCTGTTGCATT</td>
<td>AGCTACGAGATGAAAAAG</td>
</tr>
<tr>
<td>BA0698</td>
<td>GTGTTAAAGGGGGAGCTTTG</td>
<td>GCCGCTGACATGACTTACAA</td>
</tr>
<tr>
<td>BA2125</td>
<td>ATCGGCGAAGATGGATTTG</td>
<td>AGCAAGCTTTGCTGTTTT</td>
</tr>
<tr>
<td>BA2748</td>
<td>ATGGCCCATAGAATGG</td>
<td>TATAATGCGCTTACCTG</td>
</tr>
<tr>
<td>BA4073</td>
<td>ATGGGCGCACTATGAT</td>
<td>GGTTTAGCTACCTG</td>
</tr>
<tr>
<td>BA4704</td>
<td>GGTGTTGCGTTTACCTTTTG</td>
<td>GCTAAGGCTGTGGGAGTGG</td>
</tr>
<tr>
<td>BA4606</td>
<td>TTAATTGCGAATGTGG</td>
<td>GCCGTCCACTTGATACAA</td>
</tr>
<tr>
<td>BA4547</td>
<td>GCCAGATTGATCAGAATGTT</td>
<td>GTGAAGATGCTAGGCCGGTG</td>
</tr>
<tr>
<td>pXO1-110 (pagaA)</td>
<td>CATGACGCTGCTTCTTGGATA</td>
<td>CATGTTCGAGCCAAAGT</td>
</tr>
<tr>
<td>pXO2-08</td>
<td>TGGAACTTCAGGGCAAATTC</td>
<td>GCCGTTTTATGCGCCCAATA</td>
</tr>
<tr>
<td>BX0048</td>
<td>TGGCTGCTAGGTTTTGCTT</td>
<td>TCTACCCCCATCCACATA</td>
</tr>
</tbody>
</table>

**Primer sets for selected paralogs/homologs**

**Paralog/similar proteins of pXO2-08**

| BA1952 | ATCTCCATCCGCTGAGATC | TCCGCACTGTTTTACGAG |
| BA5427 | ACAAGAAGCTGACACAAAA | CTTGCTGCTGTTCTGTTT |

**Paralog/similar proteins of pXO473**

| pXO2-42 (armA) | TACCACCTGACGGCAACAA | GCATCGTGAATGCGGTT |
| pXO485 | ACAGTTTGGTGCTAGTGGT | CGCCATACCGAAGGTAGT |
| BA2446 | AAATCGTGGTCCGAGAGGAC | TTTAGATGCGGGGATTGCT |
| BA2805 | GCCGGTCTTGTCTCTACGT | AACGCCTCCCTAACCATCCTG |
| BA3737 | GACAGGATTTGCCGATATTAGGG | CACTATGAGTCCACAA |

After an initial denaturation at 95 °C for 3 min, the RT-PCR cycle was as follows: denaturation at 94 °C for 30 sec, extension at 58 °C for 30 sec, extension at 72 °C for 1 min, followed by a plate read. The cycle was repeated for a total of 40 cycles. C(t) values were set at 1 min, followed by a plate read.

The cycle was repeated for a total of 40 cycles. C(t) values were set at 1 min, followed by a plate read.

**References**


**Functional activity of exogenous BA0485 and BA2446 recombinant amidase proteins on vegetative cell suspensions of Bacillus sp. and E. coli**

To begin to determine the effect of identified amidases on B. anthracis cell wall integrity, we incubated a 1:40 dilution in LB broth of overnight cultures of B. anthracis Sterne strain, B. cereus ATCC 14579, B. subtilis 168, and E. coli DH5α for 2–2.5 hrs in an environmental shaker at 37 °C. We centrifuged cultures, resuspended bacteria to an A595 of 0.4 in 20 mM sodium phosphate buffer (pH 4.7, 7.0, and 8.7), added purified BA0485 or BA2446 protein to a final concentration of 2 μM to cell suspensions, and measured A595 every 2 min. Control samples contained buffer lacking recombinant protein. We quantified viable bacterial CFUs using LB agar plates prior to addition of BA0485 or BA2446 protein, and then again after 20 min of incubation. We assessed cellular morphology via Gram stain and light microscopy, and calculated mean vegetative cell chain length based on ten fields of view.

**Biosafety**

All work described in this experiment involving B. anthracis strains listed in the Centers for Disease Control and Prevention Select Agent Program was performed in fully-certified BL2 and BL3 facilities, was fully approved, and met all institutional and local, state, and federal governmental regulations. Procedures that required BL3 animal work involving macaques were performed through the Centers for Disease Control and Prevention; procedures that required BL3 animal work involving mice were performed through the University of New Mexico Health Science Center, Albuquerque, NM.

**Author Contributions**

Conceived and designed the experiments: SC TK ER SR AP MD MH JH. Performed the experiments: SR AP JY MD AB MB. Analyzed the data: SC TK ER SR AP MD MH JH. Contributed reagents/materials/analysis tools: TK SR AP MD. Performed the experiments: TK ER SR AP MD MH JH. Wrote the paper: ER SR.


