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Identification of a Protein Subset of the Anthrax Spore Immunome in Humans Immunized with the Anthrax Vaccine Adsorbed Preparation

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We identified spore targets of Anthrax Vaccine Adsorbed (AVA)-induced immunity in humans by screening recombinant clones of a previously generated, limited genomic *Bacillus anthracis* Sterne (pXO1$^+$, pXO2$^-$) expression library of putative spore surface (spore-associated [SA]) proteins with pooled sera from human adults immunized with AVA (immune sera), the anthrax vaccine currently approved for use by humans in the United States. We identified 69 clones that reacted specifically with pooled immune sera but not with pooled sera obtained from the same individuals prior to immunization. Positive clones expressed proteins previously identified as localized on the anthrax spore surface, proteins highly expressed during spore germination, orthologs of proteins of diverse pathogens under investigation as drug targets, and orthologs of proteins contributing to the virulence of both gram-positive and gram-negative pathogens. Among the reactive clones identified by this immunological screen was one expressing a 15.2-kDa hypothetical protein encoded by a gene with no significant homology to sequences contained in databases. Further studies are required to define the subset of SA proteins identified in this study that contribute to the virulence of this pathogen. We hypothesize that optimal delivery of a subset of SA proteins identified by such studies to the immune system in combination with protective antigen (PA), the principal immunogen in AVA, might facilitate the development of defined, nonreactogenic, more-efficacious PA-based anthrax vaccines. Future studies might also facilitate the identification of SA proteins with potential to serve as targets for drug design, spore inactivation, or spore detection strategies.

Bacillus anthracis is a facultatively anaerobic, nonmotile, gram-positive, endospore-forming bacillus, which primarily causes a fatal disease in herbivores (51). Human infection is acquired upon exposure to endospores, and depending on the route of infection, the disease may manifest as cutaneous (least dangerous and easily treatable), inhalational (often fatal), or gastrointestinal (rare) anthrax (43, 51). Irrespective of the route of infection, progression to systemic disease can occur. Endospores phagocytosed by macrophages are transported to the regional lymph nodes, where they germinate into vegetative bacilli (43, 51), which then multiply in the lymphatic system and disseminate into the bloodstream, causing massive septicemia. The organism then elaborates virulence factors that cause a variety of systemic effects leading to the death of the host (43, 51).

Thus far, the pathogenicity of *B. anthracis* has been attributed to the production of virulence factors encoded on two virulence plasmids, pXO1 and pXO2, which are present in all fully virulent strains. pXO2 encodes an antiphagocytic γ-D-glutamic acid capsule. pXO1 encodes three virulence proteins—protective antigen (PA), lethal factor (LF), and the edema factor (EF)—which assemble to form two binary toxins. PA, the nontoxic, receptor-binding moiety, can assemble either with EF, to form edema toxin (ET), or with LF, to form lethal toxin (LT). The enzymatic moiety of ET is an adenylate cyclase (51) that acts by increasing intracellular levels of cyclic AMP, which is responsible for the edema typical in patients with cutaneous anthrax. The enzymatic moiety of LT is a zinc metalloprotease (51) that exerts its effect by cleaving mitogen-activated protein kinase kinase. The precise mechanism by which LT causes death in systemic anthrax is still under investigation. Results of recent studies with mice implicate hypoxia-induced tissue injury (50) and genetic factors (49) in LT-mediated lethality rather than induction of proinflammatory cytokines as suggested earlier (51). In addition, results of several recent studies have alluded to other, unidentified virulence determinants acting in concert with the factors mentioned above to play a contributory role in anthrax pathogenesis (16, 20, 45, 59, 74, 78).

Although therapeutic options are available to successfully treat the syndromes of anthrax upon early diagnosis, vaccination may be the most effective strategy to thwart the disease (43), especially for target populations likely to be exposed to anthrax spores, such as military personnel and workers in the wool and leather industries. Vaccination also remains the most economical means of mass immunization. The anthrax vaccine currently approved for human use in the United States, Anthrax Vaccine Adsorbed (AVA), is a cell-free filtrate prepared from a formalin-treated culture supernatant of a nonproteolytic, toxigenic, unencapsulated, avirulent *B. anthracis* strain (pXO1$^+$ pXO2$^-$), V770-NP1-R, adsorbed to the adjuvant aluminum hydroxide (36). It is administered subcutaneously in a

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volume of 0.5 ml at 0, 2, and 4 weeks and at 6, 12, and 18 months. Thereafter, boosters administered annually are essential to maintain protective immunity (25, 43). A similar vaccine, prepared by adsorbing a sterile culture supernatant filtrate of the $32F_2$ Sterne strain to potassium aluminum sulfate, is licensed for use in the United Kingdom (43, 79). Studies have demonstrated that AVA is safe (36) and protects against both cutaneous (36, 43) and inhalational (25, 36, 43) anthrax.

Despite documentation attesting to the safety and efficacy of AVA, anthrax vaccines currently approved for human use have several limitations. Immunization with human-use acellular, PA-based vaccines reportedly induces low and transient immune responses (28, 44), and consistent with this observation, multiple administrations of AVA are required for induction of protective immunity (15). Immunization is associated with local and sometimes systemic reactogenicity, attributable to residual LF and EF, which may combine with PA to form active LT and ET; the adjuvant used; and also the presence of uncharacterized components in vaccine preparations (36, 77, 79). Additional limitations of AVA include the lack of standardization in the manufacturing process, resulting in batch-to-batch differences in the amount of PA, and the unavailability of reliable assays to measure the potency of vaccine preparations (43).

The drawbacks of AVA present a strong rationale for the development of a defined anthrax vaccine that is free of adverse effects and capable of inducing sustained, long-term protective immunity. Toward development of such vaccines, it has been thought that better protection could be achieved by targeting early infection (to prevent septicemia) as well by targeting the toxins elaborated (to prevent toxemia) (16, 20). Studies have therefore focused on the use of anthrax spores to augment the protective efficacy of PA-based vaccines. Cohen et al. (20) demonstrated that a single subcutaneous administration of 5×10^7 spores of a live, attenuated *B. anthracis* vaccine strain ($pXO1^-$ $pXO2^-$; a derivative of the nonproteolytic strain V770-NP1-R expressing chromosomally encoded PA under the control of the constitutively active α-amylase promoter of *Bacillus subtilis*) afforded complete protection to guinea pigs against a lethal challenge from wild-type anthrax spores, whereas the same vaccine administered as vegetative cells conferred only partial protection (20). Significantly, the spore-based vaccine elicited extremely high anti-exosporium antibody titers and approximately fivefold-higher neutralizing anti-PA antibody titers than the same vaccine administered as vegetative cells, suggesting that the higher protective efficacy of the spore-based vaccine was due to the contribution of unidentified spore antigens as well as to better presentation of PA to the immune system (20). Brossier et al. (16) provided compelling evidence that the administration of a combination vaccine composed of purified PA and 10^8 formaldehyde-inactivated spores of a *B. anthracis* strain that carried point mutations within catalytic regions of both LF and EF completely protected both guinea pigs and mice from a lethal challenge with wild-type anthrax spores, whereas a vaccine based on purified PA alone conferred only partial protection (16). Importantly, the PA-neutralizing activities of the two forms of the vaccine were similar (16), suggesting that the protection conferred by the combination vaccine was not due to anti-PA antibodies alone but also to the contribution of antibodies directed against unidentified spore antigens. Similar observations have been reported from several other studies as well (35, 45, 59, 74).

In addition to the above, recent experimental evidence suggests that unidentified antigens, especially those on the anthrax spore, might contribute to protective immunity engendered by AVA. Welkos et al. (78), using immune sera from monkeys vaccinated with AVA, demonstrated that in addition to PA, unidentified anthrax spore surface antigens might also be targets of AVA-induced protective immunity. They showed that the ability of immune sera from such monkeys to enhance phagocytosis of spores by murine peritoneal macrophages was in part due to the humoral immune response against unidentified spore antigens (78). Indirectly supporting these observations were those of another recent study that utilized proteomic analysis to examine the composition of the anthrax vaccine approved for human use in the United Kingdom (79). Results indicated the presence of previously identified anthrax spore surface proteins such as enolase, nucleoside diphosphate kinase, fructose-bisphosphate aldolase (class II), and the 60-kDa heat shock chaperonin GroEL (46) in vaccine preparations, although the immune response to these components was not examined (79).

The impetus for the present study was provided by the above observations as well as the fact that the protein makeup of the spore, especially that of the surface (which is the structure likely to interact with the immune system), is complex and composed of diverse proteins (46). We therefore reasoned that the protective immune response induced by multiple administrations of AVA is likely to be directed against several heretofore-unidentified spore proteins. We considered that identification of such proteins might facilitate the future development of defined, more-efficacious acellular PA-based vaccines and might also facilitate the development of drugs as well as spore inactivation and spore detection strategies. Therefore, we examined the reactivities of 292 recombinant clones that constituted a limited expression library of putative anthrax spore surface (spore-associated [SA]) proteins generated in a recent study in our laboratory (unpublished data) with sera obtained from two human adults immunized with AVA (immune sera). We report the identification of proteins expressed from 69 positive clones that reacted with immune sera but not with sera obtained from the same individuals prior to vaccination (preimmune sera). This, to our knowledge, is the first report of the identification of a set of proteins that are part of the anthrax spore immunome in humans immunized with AVA.

MATERIALS AND METHODS

Generation of a limited expression library of anthrax spore-associated proteins. An inducible *B. anthracis* genomic DNA expression library was first constructed using genomic DNA isolated from the nonpathogenic *B. anthracis* strain Sterne in the pET30 (abc) series of expression vectors (which permit cloning of inserts in each of three reading frames under the control of the T7 phage promoter) and the expression host *Escherichia coli* BL21(DE3) (Novagen, Madison, WI). A limited expression library of putative anthrax spore surface (spore-associated) proteins was then generated by screening the above genomic expression library with affinity-purified polyclonal antibodies generated in goats against a mixture of gamma-irradiated, purified, intact spores produced by *B. anthracis* strains Vollum, Ames, and Sterne (Chemicon, Temecula, CA). A total of 292 reactive clones were identified (unpublished data) and constituted the limited

expression library of anthrax spore-associated proteins that was probed with sera from AVA-vaccinated humans (see below) in this study.

Preimmune and immune human sera. Preimmune and immune serum samples were collected from two human adult volunteers immunized with AVA at the Division of Infectious Diseases, Massachusetts General Hospital, Boston. The institutional review board of the Massachusetts General Hospital approved the collection and use of these serum samples. Specifically, serum samples (10 ml) were collected prior to the first administration (preimmune sera) and 2 weeks following the fourth administration (dose administered at 6 months) of AVA (immune sera). We utilized sera from this time point as a probe for our screen, since results of experiments with nonhuman primates indicate that protective immunity against inhalational anthrax is engendered following two administrations of AVA (25). Serum samples were dispensed in small volumes and stored at −70°C until use.

Preparation of preimmune and immune sera for screening of the limited expression library of anthrax spore-associated proteins. Prior to use as probes, sera were pooled to compensate for differences in immune responses of individuals and to identify a wider array of reactive spore-associated proteins. Sera were used either directly (crude sera) or following affinity purification (affinity-purified sera). Sera were affinity purified using magnetic beads linked to either protein A or protein G (Dynabeads protein A or Dynabeads protein G, respectively) according to the manufacturer's instructions (Dynal Biotech, Lake Success, NY) with modifications. Protein A reportedly binds all human immunoglobulin (Ig) isotypes and IgG subclasses except IgG3, whereas protein G binds all IgG subclasses but not other Ig isotypes (29). We initially affinity purified pooled sera using both Dynabeads protein A and Dynabeads protein G; however, pilot colony immunoblotting experiments revealed that the pooled sera affinity purified using Dynabeads protein A consistently yielded better results (data not shown), and these affinity-purified sera were therefore used as a probe in subsequent colony immunoblotting experiments. For capture of antibodies by Dynabeads protein A, 10 μl of pooled preimmune or immune sera was added to 100 μl of beads, prepared according to the manufacturer's instructions, and incubated at room temperature with slow tilt rotation for 30 min. The beads were then pulled down using a magnet, the supernatant decanted, and beads washed according to the manufacturer's instructions to remove loosely bound components. Specifically bound Igs were eluted with 0.1 M citrate (pH 3.0) directly into 1 M Tris (pH 9.0). Crude and affinity-purified sera were stored at 4°C following the addition of 0.02% sodium azide until further use. Long-term storage was in 50% glycerol at −70°C.

Assessment of the quality of pooled sera. We assessed the quality of crude and affinity-purified sera by reacting pooled preimmune and immune sera with a recombinant (test) clone, *E. coli* BL21(DE3)(pSMR-PA), expressing full-length PA, by utilizing a colony immunoblot assay. We examined reactivity against this particular protein because PA reportedly is the principal immunogen and a major component of AVA (43), and anti-PA antibodies are a gauge of the host response to immunization (36). For immunoscreening, the test clone and *E. coli* BL21(DE3)(pET30a) (negative control) were toothpicked onto duplicate Luria-Bertani (LB) plates supplemented with 50 μg/ml of kanamycin (LB-Kan) and incubated overnight at room temperature. Colonies were lifted from one of the plates (the other plate constituted the "master" plate) by using a nitrocellulose filter and were placed colony side up on a fresh LB-Kan plate containing 1 mM isopropyl-β-D-thiogalactoside (IPTG). Following an overnight incubation at 30°C to induce expression of genes contained within cloned inserts, colonies on plates were partially lysed by exposure to chloroform vapors for 15 min in a candle jar. The filters were then removed from the plates, air dried, and blocked using 5% nonfat milk in phosphate-buffered saline (pH 7.4) (PBS) for 1 h at room temperature. After a rinse with PBS containing 0.05% Tween 20 (PBS-T), filters were probed either with a 1:5,000 dilution of pooled crude preimmune or immune sera or with a 1:500 dilution of pooled affinity-purified preimmune or immune sera. Following an overnight incubation at 4°C on a rocking platform, filters were washed three times with PBS-T and incubated with a 1:20,000 dilution of peroxidase-labeled goat IgG raised against the human gamma globulin fraction (ICN/Cappel, Aurora, OH). Filters were developed using an ECL chemiluminescence kit (Amersham Biosciences), and positive clones were identified by their positions on the "master" plate.

Screening of the limited, expression library of anthrax spore-associated proteins by colony immunoblotting. Prior to screening, each of the 292 clones expressing spore-associated proteins was toothpicked onto duplicate LB-Kan plates in a grid pattern alternating with the negative control and incubated at 37°C for 6 h. Colonies were lifted, and induction of gene expression from cloned inserts was performed, as described above. The filters were processed as described above and probed with a 1:10,000 dilution of pooled crude preimmune or immune sera at 37°C for 1 h. Filters were then washed three times with PBS-T and incubated with a 1:20,000 dilution of peroxidase-labeled goat IgG raised against human gamma globulin fraction (ICN/Cappel) for 1 h at 37°C. Filters were washed and developed as before, and reactive clones were identified by their positions on the "master" plate. Positive clones were purified and reactivity confirmed via an additional round of colony immunoblotting using pooled affinity-purified preimmune and immune sera at a dilution of 1:500 and using the procedure described in the preceding section for screening the test clone and the negative control.

To identify proteins expressed from each clone, lysates of each positive clone were prepared as described previously (41) and used as templates in PCR. Amplification reactions were performed using vector-specific primers obtained from the DNA Synthesis Core Facility, Department of Molecular Biology, Massachusetts General Hospital, as described previously (41). Amplicons were purified using the QIAQuick PCR purification kit (QIAGEN, Valencia, CA) and subjected to DNA sequencing at the DNA Sequencing Core Facility, Department of Molecular Biology, Massachusetts General Hospital, using ABI Prism DiTerminator cycle sequencing with AmpliTaq DNA polymerase FS and an ABI 377 DNA sequencer (Perkin-Elmer Applied Biosystems Division, Foster City, CA).

Genes on cloned inserts within reactive clones were identified via BLAST by comparing nucleotide sequences against those contained in the nonredundant database at the National Center for Biotechnology information (http://www.ncbi.nlm.nih.gov/BLAST) and against sequences of *B. anthracis* strain Ames at The Institute for Genomic Research (TIGR) (http://www.tigr.org). Protein identities and functions (see Table 1) were determined from the TIGR database, the Swiss-Prot/trEMBL databases (http://us.expasy.org/sprot/), or the Conserved Domain Database (CDD) (48) at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

RESULTS AND DISCUSSION

Pooled immune sera react specifically with a recombinant clone expressing full-length PA. The documentation that human-use acellular PA-based vaccines induce weak and inconsistent immune responses (28, 44), coupled with the lack of standardization of the vaccine manufacturing process (43), dictated that we examine the quality of pooled immune sera prior to their use as a probe for screening a previously generated limited expression library of anthrax spore-associated proteins. As shown in Fig. 1, the recombinant clone expressing full-length PA was strongly reactive with pooled crude and affinity-purified immune sera but not with pooled crude or affinity purified preimmune sera, indicating a robust immune response to AVA. That this reactivity was specific was also evidenced by the result that neither the crude nor the affinity-purified immune serum pool reacted with the negative control, which consisted of the *E. coli* BL21(DE3) host strain carrying the native, nonrecombinant expression plasmid vector pET30a (the same host-vector combination used in the construction of the *B. anthracis* expression library). These results indicated that the pooled preimmune and immune sera were suitable for probing the limited expression library of anthrax spore-associated proteins.

Anthrax spore-associated proteins reactive with immune sera. Prompted by previous reports that unidentified antigens, including those that were spore associated, might significantly contribute to the protective immunity of PA-based vaccines (16, 20, 45, 59, 74, 78) and by the documentation that immunization with AVA induces protective immunity against both cutaneous (36, 43) and inhalational (25, 36, 43) anthrax, albeit following multiple administrations, we investigated whether a subset of the 292 library clones expressing anthrax spore surface proteins, previously identified in our laboratory, might be part of the *B. anthracis* immunome in patients immunized with AVA. We did this by examining the reactivities of recombinant

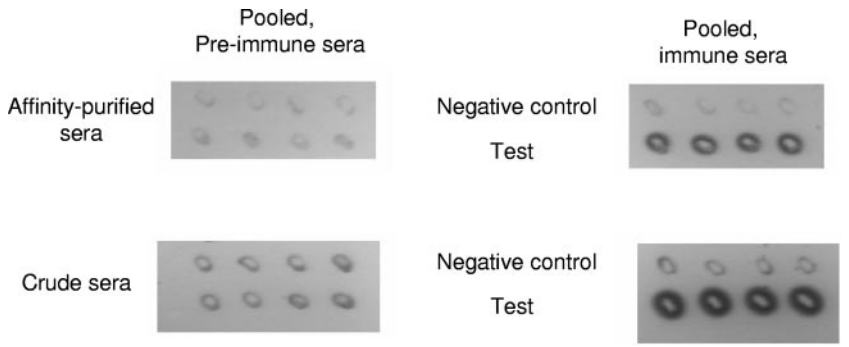

FIG. 1. Colony immunoblot assay reactivities of pooled preimmune and immune sera with a test clone consisting of *E. coli* BL21(DE3)(pSMR-PA) expressing full-length PA and with a negative control consisting of the expression host strain *E. coli* BL21(DE3) carrying the native plasmid, pET30a.

clones expressing these proteins with pooled crude and affinity-purified preimmune and immune sera from two human adult volunteers administered four doses of AVA. This immunological screen resulted in the identification of 69 expression library clones expressing proteins that were targets of AVA-induced immunity. The spore-associated proteins expressed from cloned inserts within such reactive clones are listed in Table 1 and described below.

The anthrax spore immunome in vaccinated humans comprised several proteins involved in protein synthesis, modification, and repair (Table 1). Included within this group were clones expressing a glutamyl-tRNA synthetase (GltS) and a seryl-tRNA synthetase (SerS), both of which catalyze the attachment of specific amino acids to cognate tRNAs (tRNA aminoacylation). Because such proteins play a central role in protein synthesis and also because they are highly conserved in all bacteria, these proteins are currently the focus of intense research as targets for the development of anti-infectives against both gram-positive and gram-negative pathogens (68). Interestingly, tRNA synthetases reportedly are present on the anthrax spore surface (46), although the precise function of such proteins in this location is unclear. Also identified was a clone expressing a polypeptide deformylase, Def-1, which catalyzes the removal of a formyl group from the N-terminal methionine of ribosomally synthesized proteins in eubacteria. Because deformylation of polypeptide chains is imperative for protein maturation, which in turn is essential for bacterial-cell viability, these proteins are also targets for the development of antimicrobial compounds (71). A particularly interesting protein expressed by a clone in this group was a unique RNA binding protein called SmpB, which binds with high affinity to a tmRNA molecule (so called because it functions both as a tRNA and as an mRNA) encoded by *ssrA* (SsrA RNA) (40) to form a complex that functions in ridding the bacterial cell of incompletely synthesized, nascent polypeptides. The essential preliminary step in this process, which involves binding of SsrA RNA to stalled ribosomes on defective mRNA in order to add a short C-terminal tag to the nascent polypeptide and earmark it for degradation by C-terminal-specific proteases, is facilitated by the high-affinity binding of SmpB to SsrA RNA (40). The function of SmpB as a spore-associated protein is unclear; however, suggesting a role in the virulence of *B. anthracis* is the observation that disruption of *smpB* attenuates the virulence of *Salmonella enterica* serovar Typhimurium in mice and reduces bacterial survival within macrophages (8). Also supporting a possible contribution to anthrax pathogenesis are the facts that deletion of *ssrA* (deletion of *smpB* in *E. coli* results in phenotypes identical to those in *ssrA*-defective cells) reportedly results in a >200-fold decrease in virulence in a murine model of typhoid fever (38) and that *ssrA* influences the expression of several *Salmonella* serovar Typhimurium genes induced specifically during host infection, a subset of which reportedly contribute to the virulence of diverse bacterial pathogens (38). In addition, because bacterial cells lacking tmRNA demonstrate increased sensitivity to inhibitors of protein synthesis (22), SmpB may also have potential as a target for drug design. Another protein identified was the peptide chain release factor I (PrfA), a small protein that directs termination of translation in response to stop codons. Mutations in *prfA* reportedly inhibit cell division (55).

Transport and binding proteins included components of the ATP-binding cassette (ABC) superfamily, as well as members of the major facilitator superfamily (MFS) (Table 1). Specifically identified in this study were clones expressing components of several ABC-type transporters involved in the uptake and transport of oligopeptides. Multicomponent primary transporters have been well characterized in both *E. coli* and *Salmonella* serovar Typhimurium and reportedly carry out diverse functions in these organisms (57). Additionally, such proteins also function in gram-positive bacteria, in sensing extracellular signaling molecules essential for the initiation of competence and sporulation in *B. subtilis* (58, 65) and promoting growth of *Listeria monocytogenes* at low temperatures and within macrophages (14). Also identified was a clone expressing a sugar transporter (specific substrate unknown) that belonged to the MFS and another clone expressing an efflux transporter of the EamA type, which in *E. coli* serves to regulate the level of metabolites by transporting excess metabolites of the cysteine pathway, which would otherwise disrupt metabolism, out of the cell (24). The identification of ABC-type transporters as components of the spore immunome was expected, since components of such transporters have been identified previously on the surface of the anthrax spore (46), although the role played by such proteins in a spore location has yet to be defined. Of potential significance were two conserved hypothetical proteins encoded by genes on inserts

TABLE 1. Anthrax spore-associated proteins reactive with sera of human adults immunized with AVA

Functional category[a] and clone no.	Locus ID[b] in:		Gene/protein/function[c]
	B. anthracis Sterne	*B. anthracis* Ames	
Protein synthesis, modification, repair			
14	BAS0087	BA0086	*gltx*/GltX; glutamyl-tRNA synthetase/tRNA aminoacylation
109	BAS3884	BA4187	*def-1*/Def-1; polypeptide deformylase/protein modification and repair
368[d]	BAS4955	BA5332	*smpB*/SmpB; ssrA-binding protein/protein synthesis: binds specifically to the *ssrA* RNA (tmRNA) and required for stable association of *ssrA* with ribosomes
	BAS4956	BA5334	*vacB*/VacB; RNase R/transcription: RNA processing
1188	BAS5178	BA5572	*prfA*/PrfA; peptide chain release factor I/translation: peptide chain release factor I directs the termination of translation in response to the peptide chain termination codons UAG and UAA
1262[d]	BAS0016	BA0013	None/none; sigma70_4 (region 4 of sigma factor 70)/binding to the −35 promoter element via a helix-turn-helix motif[e]
	BAS0015	BA0012	*serS*/SerS; seryl-tRNA synthetase/tRNA aminoacylation
Transport and binding			
103[d]	BAS0206	BA0210	None/none; transporter, EamA family/transport and binding of proteins
	BAS0205	BA0208	None/none; transcriptional regulator, LysR family/DNA interactions
268	BAS1105	BA1195	None/none; oligopeptide ABC transporter, ATP binding protein/transport and binding of amino acids, peptides, and amines
373[d]	BAS3376	BA3641	None/none; rADc (rRNA adenine dimethylase)/methylation of an adenine of rRNA[e]
	BAS3377	BA3642	None/none; oligopeptide ABC transporter, oligopeptide binding protein/transport and binding of amino acids, peptides, and amines
824	BAS4394	BA4734	None/none; oligopeptide ABC transporter, ATPbinding protein/transport and binding of amino acids, peptides, and amines
1077[d]	BAS4648	BA5003	None/none; ABC transporter, putative ATP binding protein/transport and binding of unknown substrates
	BAS4647	BA5002	None/none; conserved hypothetical protein (putative rRNA methylase)/rRNA methylation
1104[d]	BAS3035	BA3268	None/none; conserved hypothetical protein/unknown
	BAS3034	BA3267	None/none; major facilitator family protein/sugar transport (specific substrate unknown)
164	BAS2639	BA2830	None/none; sodium:alanine symporter family protein/ion/amino acid transport
Cell envelope			
151	BAS1932	BA2079	*dal-2*/Dal-2; alanine racemase/biosynthesis of murein sacculus and peptidoglycan
232	BAS5205	BA5604	None/none; LPXTG motif cell wall anchor domain protein containing a collagen binding domain/unknown
367	BAS5183	BA5578	*murA2*/MurA2; UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase 2/biosynthesis of murein sacculus and peptidoglycan
380	BAS5217	BA5615	None/none; membrane protein PfoR component of the sugar phosphotransferase system, sucrose and fructose specific/carbohydrate transport and metabolism
545	BAS1477	BA1593	None/none; putative membrane protein/unknown
739	BAS1135	BA1228	None/none; glucose-1-phosphate thymidylyltransferase, putative/biosynthesis and degradation of surface polysaccharides and lipopolysaccharides
862	BAS5285	BA5681	None/none; membrane protein, putative/unknown
812[d]	BAS0638	BA0672	*inhA*/InhA; immune inhibitor A metalloprotease/metallopeptidase functioning in proteolysis, peptidolysis
	BAS0637	BA0670	None/none; transaldolase/functions in the pentose phosphate pathway
355	BAS1246	BA1346	None/none; internalin, putative/pathogenesis
239	BAS4444	BA4789	None/none; LPXTG motif cell wall anchor domain protein containing a "NEAT" domain/unknown
Sporulation and germination			
435	BAS4338	BA4672	*obG*/ObG; Spo0B-associated GTP binding protein/sporulation and germination
528	BAS4236	BA4566	*sigK*/SigK; transcription factor/sporulation and germination
Metabolism			
19 (identified twice)	BAS4413	BA4754	*sdhA*/SdhA; succinate dehydrogenase, flavoprotein subunit/tricarboxylic acid cycle
195	BAS2768	BA2980	None/none; carbohydrate kinase, FGGY family, authentic frameshift/sugar metabolism
243[d]	BAS0672	BA0706	None/none; *N*-acyl-L-amino acid amidohydrolase (peptidase family M20)/metabolism of amino acids and amines
	BAS0673	BA0707	None/none; conserved hypothetical protein
255	BAS4315	BA4650	*ruvB*/RuvB; DNA replication, recombination, and repair
404	BAS5149	BA5541	*nuoB*/NuoB; NADH dehydrogenase I, B subunit/electron transport
777	BAS4186	BA4508	*nfo*/Nfo; endonuclease IV/DNA replication, recombination, and repair
829[d]	BAS4875	BA5246	None/none; acyl-CoA dehydrogenase/degradation of fatty acids and phospholipids
	BAS4876	BA5248	None/none; acetyl-CoA acetyltransferase/fatty acid and phospholipid metabolism
1091	BAS2724	BA2932	None/none; putative glutathionylspermidine synthase/polyamine biosynthesis
1111[d]	BAS1283	BA1385	None/none; 2-nitropropane dioxygenase/nitrogen metabolism (oxidoreductase activity)

Continued on following page

TABLE 1—*Continued*

Functional category[a] and clone no.	Locus ID[b] in:		Gene/protein/function[c]
	B. anthracis Sterne	*B. anthracis* Ames	
	BAS1282	BA1384	None/none; peptidase_U61, LD-carboxypeptidase/hydrolysis of peptide bond between a dibasic amino acid and the C-terminal D-alanine in the tetrapeptide moiety in peptidoglycan; murein recycling[e]
1264	BAS4563	BA4918	*acuC*/AcuC; acetoin utilization protein/acetoin catabolism
165	BAS4985	BA5364	*eno*/Eno; enolase/glycolysis/gluconeogenesis
Amino acid biosynthesis			
218 (identified twice)	BAS0331	BA0346	None/none; 5-methylthioribose kinase, putative/biosynthesis of aspartate family
341[d]	BAS4039	BA4354	*argJ*/ArgJ; glutamate *N*-acetyltransferase/amino acid acetyltransferase/biosynthesis of glutamate family
	BAS4040	BA4355	*argC*/ArgC; *N*-acetyl-gamma-glutamyl-phosphate reductase/biosynthesis of glutamate family
756	BAS1676	BA1811	*dapG-1*/DapG-1; aspartate kinase, monofunctional class/biosynthesis of aspartate family
Nucleoside/nucleotide biosynthesis			
234	BAS3739	BA4027	*pyrC*/PyrC; dihydroorotase/pyrimidine ribonucleotide biosynthesis
22	BAS4303	BA4638	*apt*/Apt; adenine phosphoribosyltransferase/salvage of nucleosides and nucleotides
387[d]	BAS5179	BA5573	*tdk*/Tdk; thymidine kinase/nucleotide and nucleoside interconversions
	BAS5180	BA5574	*rpmE*/RpmE; ribosomal protein L31/synthesis and modification of ribosomal proteins
1284 (identified twice)	BAS0286	BA0299	*purD*/PurD; phosphoribosylamine-glycine ligase/purine ribonucleotide biosynthesis
Biosynthesis of cofactors, prosthetic groups, and carriers			
26	BAS1986	BA2134	None/none; molybdopterin biosynthesis protein, putative/molybdopterin biosynthesis
1295	BAS5244	BA5463	*thiC*/ThiC; thiamine biosynthesis protein/thiamine biosynthesis
753	BAS0698	BA0732	*thiG*/ThiG; thiazole biosynthesis protein/thiamine biosynthesis
	BAS0697	BA0731	*thiS*/ThiS; sulfur transfer protein/thiamine biosynthesis
1277	BAS1423	BA1534	*menG*/MenG; 2-heptaprenyl-1,4-naphthoquinone methyltransferase/biosynthesis of menaquinone and ubiquinone
Protein degradation			
223 (identified twice)	BAS4052	BA4368	*pepT-2*/PepT-2; peptidase T/degradation of proteins, peptides, and glycopeptides
304	BAS5208	BA5606	None/none; aminopeptidase, putative/degradation of proteins, peptides, and glycopeptides
369	BAS2981	BA3206	None/none; amidohydrolase family protein, peptidase family M20/M25/M40/degradation of proteins, peptides, and glycopeptides[e]
72	BAS0167	BA0165	None/none; prolyl oligopeptidase family protein, putative (dipeptidyl peptidase type IV)/degradation of proteins, peptides, and glycopeptides
Regulatory proteins			
1031	BAS4706	BA5067	None/none; sensory box histidine kinase/protein interactions, component of two-component signal transduction system
1152	BAS4548	BA4902	None/none; transcriptional regulator, LysR family/DNA interactions
Other functions			
398	BAS3747	BA4035	*divIVA*/DivIVA; cell division initiation protein DivIVA/cell division
1284 (identified twice)	BAS0803	BA0843	None/none; catalase/detoxification
Unknown function			
80[d]	BAS2069	BA2225	None/none; acetyltransferase, GNAT family
	BAS2071	BA2226	None/none; hypothetical protein (histidinol-phosphatase, N-terminal)/amino acid transport and metabolism[e]
746	BAS4590	BA4946	None/none; conserved hypothetical protein (DUF84 family)
239	BAS3944	BA4253	None/none; hydrolase, carbon-nitrogen family
473	BAS2375	BA2552	None/none; carboxyltransferase domain protein, biotin carboxylase activity
925[d]	BAS0485	BA0514	None/none; chlorohydrolase family protein (metallodependent hydrolases, subgroup C)
	BAS0484	BA0513	None/none; RimL family of acetyltransferases/acetylation of N-terminal serine of 30S ribosomal subunit protein L7; acetyltransferase[e]
941a	BAS2700	BA2899	None/none; aminotransferase, classes I and II
941b	BAS2061	BA2217	None/none; hydrolase, alpha/beta fold family, aminopeptidase
1038	BAS4188	BA4510	None/VrrA protein
345	BAS3788	BA4077	None/none; reovirus sigma C capsid protein[e]
1272	BAS5123[f]	BA5515[f]	None/none; hypothetical protein

[a] Based on the TIGR database grouping of proteins of the sequenced *B. anthracis* strain Ames.
[b] ID, identification.
[c] Functions of the proteins identified are as designated in the TIGR database and/or in Swiss-Prot.
[d] Two genes present on the same cloned insert.
[e] Putative functions of conserved hypothetical proteins were determined using the CDD.
[f] Encoded by a gene with no significant homology to database entries.

within clone 373 and clone 1077 (Table 1), both of which were predicted by the CDD to have *S*-adenosylmethionine (SAM)-dependent methyltransferase activity. SAM-dependent methyltransferases catalyze the transfer of the methyl group from SAM to nitrogen, carbon, or oxygen and reportedly regulate diverse biological functions, including protein expression, signal transduction, and pathogenesis (10). Rounding off this group was a clone expressing an integral membrane protein of the sodium:alanine symporter family. Although L-alanine is a documented spore germinant (34, 76), it is currently unclear whether this symporter plays a role in spore germination following host infection. More studies are warranted to address this issue.

Cell envelope proteins (Table 1) included orthologs of proteins implicated in the pathogenesis of other gram-positive organisms. The screen identified clones expressing proteins possessing the C-terminal LPXTG motif, a sorting signal that anchors proteins to the cell envelope through the action of a membrane-bound cysteine protease called sortase (42). Cell wall-anchored proteins reportedly contribute to the virulence of gram-positive pathogens (81) and hence merit attention, since they might also play a role in *B. anthracis* virulence. The screen identified a clone expressing a putative internalin (InlA) (two paralogs, namely, BA1346 and BA0552, are present in the sequenced *B. anthracis* strain Ames), which in the intracellular, food-borne pathogen *L. monocytogenes* mediates invasion of nonphagocytic eukaryotic cells by binding to the extracellular domain of E-cadherin and also promotes the translocation of *L. monocytogenes* across the intestinal epithelial-cell barrier (60). Such spore-associated proteins may facilitate heretofore unidentified interactions between the anthrax spore and its environment and therefore are likely candidates for both vaccine and drug development. In addition, two other clones expressing LPXTG domain-containing proteins were identified. The open reading frame of one of these (BAS5205/BA5604 [Table 1]) was disrupted but nevertheless included a collagen-binding domain. Since collagen is a primary component of the mammalian extracellular matrix, such proteins could facilitate attachment and interaction of vegetative bacilli or spores to host connective tissues. The other LPXTG-containing protein contained a domain that is found in the vicinity of Fe^{3+} siderophore transporters and is called the "NEAT" (near transporter repeat) domain (4). Because of the association of NEAT domains with transporters functioning in iron acquisition and transport, a requisite for survival within the mammalian host, such proteins may play a major role in disease pathogenesis. Two clones expressing cell envelope proteins that are currently targets for the development of novel antimicrobials were also identified. The first was a UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase 2 (MurA2) essential for the conversion of UDP-*N*-acetylglucosamine into precursors for murein for peptidoglycan cell wall biosynthesis (11). The other was a putative glucose-1-phosphate thymidylyltransferase involved in the synthesis of dTDP-L-rhamnose, a precursor of L-rhamnose, which is a component of surface structures of both gram-positive and gram-negative bacteria such as cell wall and capsular antigens known to modulate virulence and mediate attachment to host tissues (13). Also identified was a clone expressing a predicted membrane protein, PfoR, related to membrane components of the fructose- and sucrose-specific phosphotransferase systems. BLAST analysis revealed the presence of orthologs in both *Bacillus cereus* and *Bacillus thuringiensis* that were annotated as possible regulatory proteins. It should be interesting to determine whether as a spore-associated protein, PfoR has a role in spore-germination. The screen identified a clone expressing alanine racemase, a component of the surfaces of anthrax spores as well as spores produced by other members of the *B. cereus* family (73). This enzyme functions in the conversion of the spore germinant L-alanine to the germinant inhibitor D-alanine (34, 76) and therefore may influence the rate of spore germination (39). The above, coupled with the observation that this spore surface protein is a target of the immune response of humans vaccinated with AVA, suggests that alanine racemase, acting in concert with other proteins, might contribute to the pathogenesis of anthrax. Of particular interest was a reactive clone that expressed one of the two paralogs in the genome of the sequenced *B. anthracis* strain Ames (64), annotated as the immune inhibitor A metalloprotease (InhA), a secreted zinc-dependent metalloprotease that is also produced by other members of the *B. cereus* family and is a component of the exosporium of the *B. cereus* spore (18). The function of InhA, which is well defined in the insect pathogen *B. thuringiensis*, is to facilitate the survival of the pathogen during infection by inactivating bactericidal proteins (attacins and cecropins) (21) that are components of the innate immune system of the insect host. Although the function of InhA in *B. anthracis* is unclear, it is plausible that this protein could function in a manner similar to that in *B. thuringiensis* to inactivate bactericidal host proteins during early infection and facilitate bacterial survival within the host, a hypothesis supported by the fact that *B. anthracis* InhA reportedly is 95% homologous to *B. thuringiensis* InhA and also by the fact that InhA expression is increased during spore germination (32). These facts, coupled with the observation that InhA is also a surface component of the exosporium of the anthrax spore (46) and a target of AVA-induced immunity in humans (as demonstrated in this study), suggest that InhA may be part of a suite of proteins that contribute to protective immunity against anthrax. Also included in this group were two clones expressing putative membrane proteins of unknown function, which merit further evaluation as virulence determinants in view of their surface-location.

The screen identified two clones expressing proteins involved in sporulation (Table 1). Proteins identified included a Spo0B-associated GTP binding protein of the Obg family, which in *B. subtilis* plays an undefined but essential role in growth and viability (52), and the RNA polymerase sigma-27 factor (SigK), which in the genus *Bacillus* complexes and directs RNA polymerase to initiate transcription of genes encoding spore outer coat proteins (23). Also identified were reactive clones expressing proteins involved in metabolism, such as the flavoprotein subunit of the membrane-bound enzyme succinate dehydrogenase (SdhA), an enzyme of the tricarboxylic acid cycle, which during aerobic growth converts succinate to fumarate. The immunogenicity of SdhA was not altogether unexpected, since the enzyme fumarate reductase, which functions during anaerobic respiration in catalyzing the same reaction in the reverse direction, namely, the conversion of fumarate to succinate, is strongly immunogenic in patients infected

with *Helicobacter pylori* (12). Fumarate reductase reportedly facilitates *H. pylori* colonization of the murine gastric mucosa and hence has been proposed to be both a novel drug target and a putative vaccine candidate (26). Interestingly, *B. subtilis* SdhA has also been demonstrated to function as a fumarate reductase (69). The screen identified several clones expressing proteins involved in the metabolism of macromolecules and energy. Particularly interesting was an enzyme involved in DNA replication, recombination, and repair called endonuclease IV. In *E. coli*, expression of this protein is part of the adaptive response to nitric oxide, which confers on this organism the ability to survive within activated macrophages (54). It is possible that as an anthrax spore surface protein, endonuclease IV functions in concert with other proteins to facilitate spore survival within macrophages. Also interesting was a clone that expressed a putative glutathionylspermidine (GSP) synthase, an important intermediate in the biosynthesis of the antioxidant trypanathione. Because this biosynthetic pathway is found in pathogenic *Trypanosoma* and *Leishmania* species but is absent in humans, and also because trypanathione reportedly protects against oxidative stress, GSP is under evaluation as a drug target (7). A clone expressing the acetoin utilization protein AcuC, which facilitates the utilization of the carbon storage compound acetoin via an undefined mechanism, was identified. In *B. subtilis*, acetoin is produced and excreted into the extracellular medium during exponential growth under nutrient-rich conditions and is reutilized during adverse conditions when other carbon sources have been depleted, such as during sporulation (33). Another clone contained an insert that included three genes. The first encoded acyl coenzyme A (acyl-CoA) dehydrogenase, an enzyme that functions in fatty acid and phospholipid metabolism and is reportedly involved in the adaptive response of *Salmonella* serovar Typhimurium to carbon starvation (72). Although deletion of the gene encoding acyl-CoA dehydrogenase did not decrease the virulence of *Salmonella* serovar Typhimurium in mice (72), the observation that this gene was specifically expressed during *Salmonella* infection of mice (47) points to the possibility that this protein may be an important component of the stress response, functioning in conjunction with other, overlapping proteins to facilitate pathogen adaptation to the in vivo environment. The second gene on the insert encoded a cytoplasmic conserved hypothetical protein, and the third gene encoded acetyl-CoA acetyltransferase, an enzyme involved in fatty acid and phospholipid metabolism. Interestingly, acetyl-CoA acetyltransferase is located on the anthrax spore surface (46). Also expressed from one of the clones in this group was an enolase functioning in glycolysis/gluconeogenesis. This enzyme is a component of the anthrax spore surface (46) and was recently reported to be a component of the anthrax vaccine approved for human use in the United Kingdom (79).

Several reactive clones expressing proteins involved in amino acid biosynthesis were identified (Table 1). Among the proteins expressed by such clones was methylribose kinase (MtnK) (identified twice), an enzyme that is highly expressed during starvation of *B. subtilis* and is involved in the recycling of methylthioribose (MTR), a degradative product of methylthioadenosine formed during the metabolism of polyamines such as spermidine and spermine (70). In *B. subtilis*, MtnK initiates recycling of MTR by phosphorylating it to MTR-1-phosphate, which then goes through a series of steps to re-form methionine (70). Because MtnK is unique to microbes (and plants), and also because it has a central role in the salvage of methionine, this enzyme is receiving attention as a drug target (27). Anthrax spore-associated MtnK might therefore be a suitable target for the development of vaccines, drugs, and/or spore inactivation agents, especially since SAM synthesized from methionine is a precursor for the synthesis of quorum-sensing signaling molecules, including autoinducer 2 (AI-2) (6, 66). A functional ortholog of the autoinducer synthase LuxS, responsible for the final step of AI-2 synthesis, was recently reported in *B. anthracis*, suggesting that this pathogen might also regulate density-dependent gene expression via AI-2 (37). Another protein expressed from a reactive clone in this group was aspartate kinase I (DapG-1), which is involved in the first step of biosynthesis of diaminopimelate from L-aspartate. Diaminopimelate is an important constituent both of the peptidoglycan of vegetative cells and of the spore cortex peptidoglycan of gram-positive bacteria, especially in members of the genus *Bacillus*. Furthermore, dipicolinate, a by-product of diaminopimelate biosynthesis, is also a part of the spore, comprising as much as 10% of the dry spore weight (19). Because aspartokinases play a pivotal role in the biosynthesis of important structural components in diverse microbes, they are the focus of efforts for the development of novel antimicrobials (63, 75).

Several reactive clones expressing proteins involved in the biosynthesis of nucleosides/nucleotides were identified (Table 1). One such protein was the dihydroorotase PyrC, which catalyzes one of the reactions in the biosynthesis of UMP from precursors such as aspartate and glutamine. A *pyrC* mutant of the gram-positive coccus *Staphylococcus aureus* is virulence attenuated in a murine model of systemic infection (9). It is likely that PyrC, as a spore component, functions in pyrimidine nucleotide synthesis during early infection, before the elaboration of toxins and other degradative enzymes that cause cellular destruction, rendering uracil and other pyrimidine nucleotides available for utilization in the pyrimidine salvage pathway (the closely related *B. subtilis* possesses a pyrimidine salvage pathway, and hence it is likely that a similar pathway also exists in *B. anthracis*). PyrC may contribute to *B. anthracis* survival within the host, since prevention of de novo pyrimidine synthesis attenuates virulence (9), and also since antibodies from humans vaccinated with AVA reacted with a clone expressing this spore-associated protein, as shown in this study. Another protein involved in the synthesis of small molecules was thymidine kinase (Tdk), which functions in pyrimidine salvage (2). A spore location suggests a possible role in salvage of thymidine derivatives from host cells/tissues for DNA synthesis, essential for multiplication of *B. anthracis* following spore germination. The same cloned insert expressing Tdk also included part of the gene encoding ribosomal protein L31, which is involved in the synthesis and modification of ribosomal proteins. Ribosomal proteins have been reported to be components of the anthrax spore surface (46). A clone was also identified expressing the monofunctional phosphoribosylamine-glycine ligase PurD (also called glycinamide ribonucleotide synthetase), an enzyme functioning in de novo purine ribonucleotide biosynthesis and reported to be required for infection of the murine lung by *Pseudomonas aeruginosa* (61).

Also in this group was a clone that expressed adenine phosphoribosyltransferase, an enzyme of the purine salvage pathway, which possibly performs a function analogous to those of the above enzymes of the pyrimidine salvage pathway.

A group of reactive clones expressed proteins involved in the biosynthesis of cofactors, prosthetic groups, and carriers (Table 1). Noteworthy among these were those that expressed proteins functioning in thiamine biosynthesis. The first was ThiC, which is expressed from a three-gene operon in *B. subtilis* and is required for the synthesis of the hydroxymethyl pyrimidine moiety of thiamine (83). The other protein identified was ThiG, an enzyme central to the synthesis of the thiazole phosphate ring of 4-methyl-5-(β-hydroxyethyl) thiazole phosphate, which is condensed with 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate to form thiamine monophosphate (56). On the same cloned insert was the gene encoding ThiS, a protein involved in sulfur transfer during thiamine biosynthesis. The fact that these enzymes function in the de novo synthesis of an important nutrient, thiamine, suggests a likely role in the in vivo survival of the pathogen and, coupled with the fact that the untranslated regions of mRNA specifying such enzymes contain a metabolite-responsive genetic control element, or "riboswitch," renders them attractive targets for drug development (80).

Several clones expressing enzymes involved in protein degradation (Table 1) were identified. One of these was a putative secreted aminopeptidase that belonged to the family of widely distributed metal-associated metalloproteases, which catalyze the removal of N-terminal amino acids from peptides and proteins. The region upstream of the gene encoding this protein has a binding site for PlcR, a pleiotropic regulator of extracellular virulence factors in closely related organisms such as *B. thuringiensis* (1, 64). Although the PlcR homolog in *B. anthracis* is truncated due to a nonsense mutation, it has been hypothesized that alternative regulatory controls may allow for PlcR-regulated proteins to contribute to *B. anthracis* virulence (64). Also, the facts that aminopeptidases are present on the anthrax spore surface (46) and have been reported to play a role in pathogenesis, particularly of intracellular parasites (53), suggest that they might play a role in the virulence of *B. anthracis*. Among other proteins expressed by reactive clones in this group was a peptidase T (PepT-2) (identified twice in this screen), a zinc metalloprotease and an amino tripeptidase, which removes the N-terminal amino acid residue from various tripeptides. Although the contributions of these proteins to the virulence of *B. anthracis* are unclear, it is of interest that PepT was one of the proteins highly expressed in *E. coli* K-12 biofilms and during growth in preconditioned medium from the laboratory strain *E. coli* DH5α (62), despite the fact that cell-to-cell signaling via acyl homoserine lactone molecules has yet to be demonstrated in gram-positive bacteria, including *B. anthracis* (6). Another spore-associated protein was a putative prolyl oligopeptidase family protein. Because members of this family, such as dipeptidyl peptidase IV, have been implicated in the virulence of certain bacterial pathogens (82), the contribution of this protein to the pathogenicity of *B. anthracis* warrants further study.

The screen identified two clones expressing regulatory proteins (Table 1). One of these was a sensory box histidine kinase component of an unknown two-component regulatory system. The fact that sensor kinases sense and transduce signals from the environment to cognate response regulator components to influence gene expression (31) renders it plausible, though speculative, that a spore surface sensor kinase might be involved in sensing the environment within the macrophage and transducing a signal via its response regulator to affect expression of genes involved in early infection. The other protein identified as part of this group was a LysR-type transcriptional regulator, which in a variety of pathogens is reportedly involved in the positive regulation of diverse classes of genes, including those encoding virulence factors (67). The screen identified another LysR-type transcriptional regulator encoded on the same insert that also encoded a transporter of the EamA family. The finding that LysR-type regulators were associated with the anthrax spore was not unexpected, since such proteins have been identified as constituents of the anthrax spore surface (46); however, the roles played by these proteins in this location have yet to be defined.

Two reactive clones expressing proteins involved in cellular processes were identified (Table 1). One of these was an uncharacterized catalase that may be part of the oxidative stress response protecting germinating spores against the lethal effects of H_2O_2, especially within phagocytic cells. It is plausible that this uncharacterized spore-associated catalase might act in conjunction with KatX, a catalase present in *B. subtilis* spores (5), and with other spore coat-resident enzymes such as superoxide dismutase to dissipate H_2O_2 and protect germinating spores against oxidative damage. The other protein in this group was a cell division initiation protein, DivIVA, which functions in the proper positioning of the septum during cell division and also promotes asymmetric septation, an essential prerequisite for sporulation (17).

The screen identified a group of clones that expressed proteins of unknown function (Table 1). Included among these was an acetyltransferase of the Gcn5-related acyltransferase (GNAT) superfamily, the members of which are widely distributed in nature and use acyl-CoA's to acylate their respective substrates. Interestingly, a paralog in the sequenced *B. anthracis* strain Ames (BA1085), which is also an acyltransferase of the GNAT superfamily, has been reported to contain the upstream binding motif for the pleiotropic positive regulator of extracellular virulence factor gene expression, PlcR (64). Also identified by the screen was a carboxyltransferase domain protein, which catalyzes the transfer of a carboxyl group from biotin to an acceptor acyl-CoA; a protein belonging to the chlorohydrolase family (a large metal-dependent hydrolase superfamily); a hydrolase of the carbon-nitrogen hydrolase family functioning in nitrogen metabolism; and an aminotransferase which catalyzes the transfer of an amino group to a cognate acceptor. Among this group of clones was one that expressed a hydrolase of the alpha/beta fold family with aminopeptidase activity, which was previously reported to be a component of the exosporium of the anthrax spore (46). Also identified was a protein encoded by *vrrA* (variable region with repetitive sequence), which encodes a 30-kDa protein in strain Sterne but encodes truncated proteins in strains Ames and Vollum, due to a single-nucleotide and a 24-bp deletion, respectively (3). Despite this, the fact the amino acid sequence of the VrrA of *B. anthracis* Sterne differs from those of the closely related *B. cereus* and *Bacillus mycoides* at 61 different positions

(3) and the fact that this protein was a target of the AVA-induced immune response in humans suggest that VrrA could be a potential virulence determinant of *B. anthracis*. Finally, the screen identified a clone expressing a 15.2-kDa hypothetical protein (BA5515) of unknown function. This hydrophilic spore-associated protein was encoded by a 360-bp gene that was present in the sequenced genomes of both *B. anthracis* strains Ames and Sterne, but not in any of the heretofore-sequenced genomes of close relatives as evidenced by BLAST analysis. Also, no significant homology to other database entries was detected.

In summary, we identified 69 clones expressing anthrax spore-associated proteins targeted by AVA-induced immunity. Positive clones expressed proteins previously identified by other methods as constituents of the anthrax spore surface, proteins highly expressed during spore germination, proteins that were orthologs of drug targets and virulence determinants of diverse pathogens, and several proteins of unknown function. It was interesting that the majority of proteins identified by this screen were not spore structural proteins but rather proteins expressed during vegetative growth; however, this was not totally unexpected, since the repertoire of the anthrax spore surface has been reported to comprise proteins expressed during vegetative growth as well as proteins expressed during sporulation (46, 78). The role of proteins expressed during the vegetative-growth phase that are also spore associated is currently unclear; however, it is plausible that when on the spore surface, these proteins take on roles (such as helping to establish early infection and spore germination) completely different from those ascribed to them during vegetative growth. Such disparate roles for the same protein at different cellular locations have been described for other pathogens as well (30).

Finally, we wish to emphasize that the functions ascribed to the above targets of AVA-induced immunity are only putative and that more-elaborate studies are essential to determine the definitive roles of these SA proteins. We have commenced the following studies toward this objective. First, because of the fact that the proteins identified in this study are associated with the infective form of this pathogen (which is likely to interact first with components of the host immune system), and also because the expression of a subset of SA proteins is reportedly increased during spore germination (32), we are employing various approaches for the identification of SA proteins operating during early infection with anthrax spores. This rationale for such experiments is further supported by documentation showing that animals immunized with AVA do not become bacteremic following challenge with virulent *B. anthracis* strains (78) and that AVA-induced immunity targets spore antigens (78), and by evidence from earlier reports that experimental spore-based anthrax vaccines protect significantly better against anthrax than those based on PA or vegetative cells alone (16, 20). Second, on the basis of the fact that a subset of SA proteins identified in our study were either orthologs of proteins of diverse pathogens under investigation as drug targets or virulence determinants of both gram-positive and gram-negative bacteria, we are in the process of generating deletions of genes encoding selected SA proteins in various *B. anthracis* strains to determine the contributions of such proteins to the virulence of this pathogen by using relevant animal models. The proteins identified by these studies will be further evaluated as an optimally delivered, PA-based vaccine for protection of appropriate animal models against challenge with virulent *B. anthracis* strains. We hypothesize that the results of such studies will help in the future development of defined, nonreactogenic anthrax vaccines. Additionally, because these proteins are part of the protein repertoire of the spore surface, a subset of which has been reported to be highly expressed during germination (32), we anticipate that the set of experiments outlined above will also help identify SA proteins with potential for the development of drugs or spore inactivation strategies. And last, on account of the spore surface localization of SA proteins and their accessibility to ligands, such as antibodies, we have initiated experiments geared toward the identification of *B. anthracis*-specific domains within SA proteins and toward confirmation of the spore surface localization of such domains for future development of assays for spore detection.

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