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Proteomic Profiling and Identification of Immunodominant Spore Antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*‡

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Differentially expressed and immunogenic spore proteins of the *Bacillus cereus* group of bacteria, which includes *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*, were identified. Comparative proteomic profiling of their spore proteins distinguished the three species from each other as well as the virulent from the avirulent strains. A total of 458 proteins encoded by 232 open reading frames were identified by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry analysis for all the species. A number of highly expressed proteins, including elongation factor Tu (EF-Tu), elongation factor G, 60-kDa chaperonin, enolase, pyruvate dehydrogenase complex, and others exist as charge variants on two-dimensional gels. These charge variants have similar masses but different isoelectric points. The majority of identified proteins have cellular roles associated with energy production, carbohydrate transport and metabolism, amino acid transport and metabolism, posttranslational modifications, and translation. Novel vaccine candidate proteins were identified using *B. anthracis* polyclonal antisera from humans postinfected with cutaneous anthrax. Fifteen immunoreactive proteins were identified in *B. anthracis* spores, whereas 7, 14, and 7 immunoreactive proteins were identified for *B. cereus* and in the virulent and avirulent strains of *B. thuringiensis* spores, respectively. Some of the immunodominant antigens include charge variants of EF-Tu, glyceraldehyde-3-phosphate dehydrogenase, dihydrolipoamide acetyltransferase, Δ-1-pyrroline-5-carboxylate dehydrogenase, and a dihydrolipoamide dehydrogenase. Alanine racemase and neutral protease were uniquely immunogenic to *B. anthracis*. Comparative analysis of the spore immunome will be of significance for further nucleic acid- and immuno-based detection systems as well as next-generation vaccine development.

The *Bacillus cereus* group of bacteria consists of genetically very closely related members that include *B. anthracis*, *B. cereus* and *B. thuringiensis* (9, 13, 21). This genetic relatedness has led to the proposal that they should be considered a single species (13). Although genetically similar, each organism occupies a different ecological niche. *B. anthracis* is the causative agent of anthrax whose spores have been used in recent bioterrorist events in the United States (30). *B. anthracis* can also cause fatal infection in domestic livestock and wreck havoc to the economy when used as a weapon for agroterrorism. On the other hand, *B. cereus* is a ubiquitous, spore-forming soil bacterium and an opportunistic human pathogen which has been implicated in food poisoning (31), whereas *B. thuringiensis* is a specific pathogen of insects that has been used as a pesticide, although a pathogenic strain has been identified (14). All three species exhibit different phenotypes and regulatory mechanisms due to various plasmid contents and pleiotropic transcriptional regulators, such as *plc*R and *atx*A (3, 29). All produce resilient spores that are covered by an outermost integument called exosporium composed of a paracrystalline basal layer and hairlike outer region (6, 12).

Vaccines have proved to be a powerful medical intervention, and recent advances in immunoproteomics have allowed the rational design of molecularly defined vaccines. Immunoproteomic approaches, such as serological proteome analysis, have provided first vaccine candidate antigens and have led to the identification of the full set of antigens or the immunome targeted by the immune system (8, 19). The comparison of multiple proteins as well as immunomes in the *B. cereus* group may allow the discovery of immunogenic structural features that are shared and conserved among these pathogens. Such features can aid in the design of a broadly protective vaccine.

The currently used anthrax vaccines in the United States and United Kingdom are based on culture filtrates or secreted proteins of avirulent *B. anthracis* strains lacking the pXO2 plasmid (22, 37). These pXO1-containing strains produce large amounts of protective antigen (PA), which is the major component of each vaccine. However, the Russian vaccine utilizes a live nonencapsulated form of *B. anthracis* spores that has been reported to have a greater efficacy than either the U.S. or United Kingdom vaccines (32). Several studies have shown that this higher efficacy is due to unidentified antigens from anthrax spores that can augment the protective efficacy of PA-based vaccines (4, 5, 38). Thus, combining PA with one of the immunodominant spore antigen(s) in a formulation may ultimately lead to a highly efficacious and safer vaccine.

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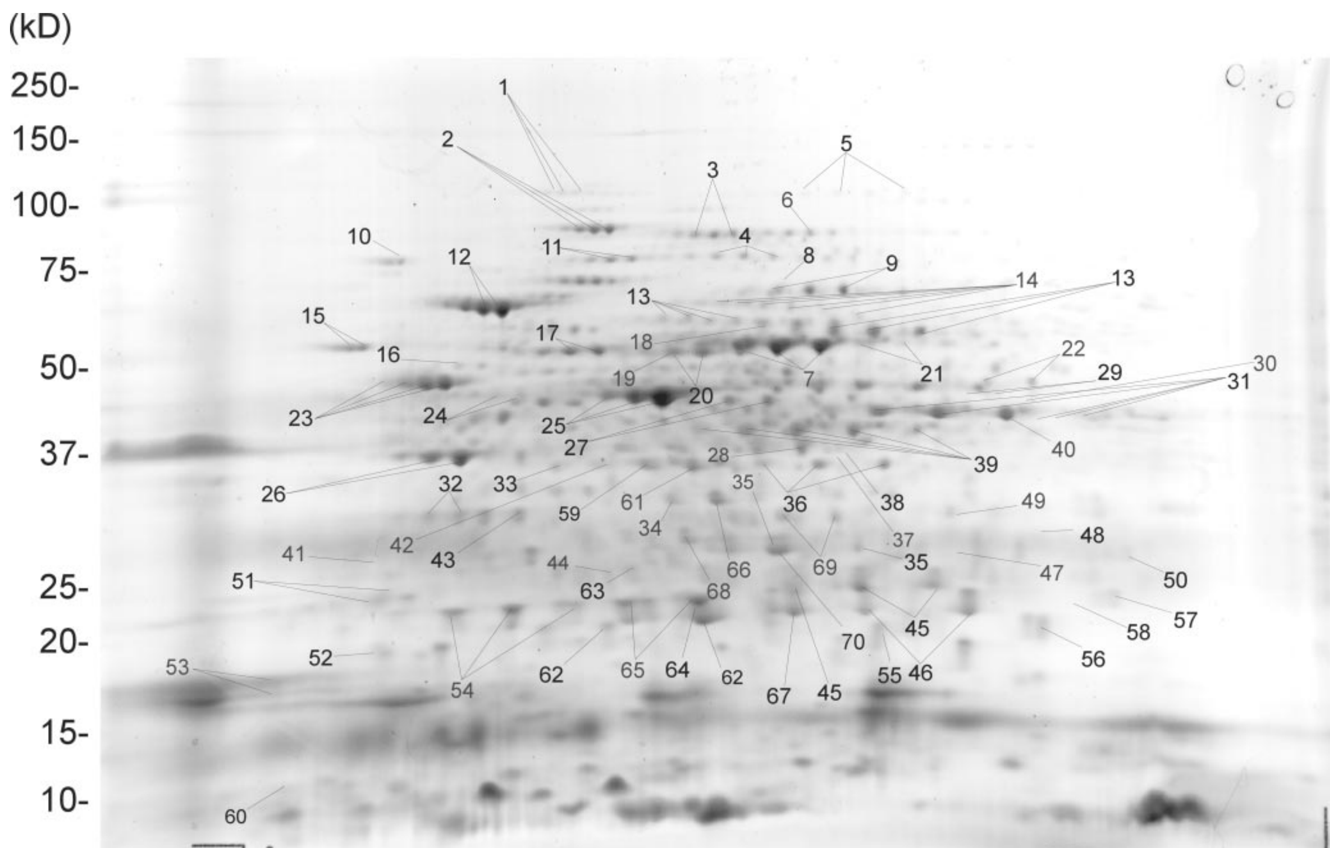

FIG. 1. Spore proteome at pH 4 to 7 of *B. anthracis* toxigenic and nonencapsulated avirulent strain RA3R.

For the development of a next-generation anthrax vaccine, an immunoproteomic analysis of the spore antigens was performed, resulting in the identification of several immunogenic proteins from *B. anthracis* and other members of the *B. cereus* group. Some of these immunogenic spore proteins can be tested as novel vaccine candidates themselves or used for enhancing the protective efficacy of a PA-based vaccine. Comparative proteomic profiling of the spores also distinguished the different species and the pathogenic from the nonpathogenic strains of the *B. cereus* group.

MATERIALS AND METHODS

Preparation and isolation of *Bacillus* spp. spore proteins. Spores of *B. anthracis* strains RA3R, A3, and 1099, *B. cereus* ATCC strain 14579, and *B. thuringiensis* strains 97-27 and ATCC 10972 were obtained by growing the *Bacillus* sp. cells on nutrient broth yeast extract agar (ATCC medium 763) at 30°C for 10 days. *B. cereus* strain 14579 and *B. thuringiensis* strain 97-27 were selected because their genomes have been annotated and are available from NCBI. The spores were isolated according to the method of Sylvestre et al. with modifications (34). The spores were collected by first wetting the surface of the plate with distilled water and scraping off the spores. The spores were then washed with distilled water five times, and the spore suspension was heated at 65°C for 30 min to kill the vegetative cells and then washed once more. The spores were purified by differential centrifugation for 30 min at 6,000 × *g* at 4°C through 50% Renografin (diatrizoate meglumine and diatrizoate sodium; Renocal-76; Bracco Diagnostic) in distilled water. The purified spores were washed three times with distilled water to remove residual Renografin. The use of a French press was omitted, and the protein extraction buffer was modified to contain 2 M thiourea instead of 8 M urea in 50 mM Tris-HCl, pH 10.00, and 2% (vol/vol) 2-mercaptoethanol. We did not use detergents and salts in the final wash to avoid losing spore surface-associated proteins, which may be important spore components, as explained previously by Liu et al. (24). An equivalent of 10^9 free spores was resuspended in 50 μl of extraction buffer, heated for 15 min at 90°C, and spun at 13,000 × *g* for 10 min. The supernatant containing the spore proteins was treated with ice-cold 10% (vol/vol) trichloroacetic acid for 30 min and spun at 13,000 × *g* for 25 min. The spore proteins were washed once with ice-cold acetone and dissolved with C7 resuspension buffer (Proteome Systems). The total protein concentration was determined using the Bio-Rad protein stain with bovine serum albumin as a standard.

2-DE and Western blot analysis. Two-dimensional gel electrophoresis (2-DE) was carried out with the ElectrophoretIQ^3 system (Proteome Systems). All supplies and reagents for 2-DE except for immobilized pH gradient (IPG) strips were purchased from Proteome Systems and used according to the manufacturer's instructions. Two hundred microliters (30 μg) of spore proteins was separated by isoelectric focusing (IEF) on 11-cm (pH 4 to 7) linear IPG strips. After 12 h of rehydration, the following focusing parameters were applied: 50 μA per strip, linear voltage increase over 8 h from 100 V to 10,000 V, and finally, 10,000 V for 10 h. After IEF, IPG strips were equilibrated in equilibration buffer and applied onto a 6 to 15% gradient sodium dodecyl sulfate-polyacrylamide gel. Gels were electrophoresed for 1.5 h at 500 V and stained with Sypro Ruby (Sigma-Aldrich, St. Louis, MO) for gel analysis or with ProteomIQ Blue for matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) analysis. Three replicate two dimensional (2-D) gels for each sample were used for computer analysis using Phoretix 2D Expression software (Nonlinear Dynamics) and MALDI-TOF MS. Following automatic spot detection on triplicate gels using Phoretix 2D Expression's detection algorithm, gels were manually warped and their common spots were matched to generate averaged gels.

Immunoblotting was conducted according to the method of Towbin et al. (35).

TABLE 1. Proteins identified in *B. anthracis* spores by using MALDI-TOF MS[a]

Spot no.	Accession no.	Theoretical mass (Da)	Mowse score	% Coverage	No. of peptides matching	Protein identification
1	gi 30263563	99,035	156	24	18	Aconitate hydratase 1
2	gi 30260298	76,333	145	27	18	Translation elongation factor G
3	gi 30263811	78,204	65	16	10	Polyribonucleotide nucleotidyltransferase
4	gi 30264669	62,234	104	23	10	Pyruvate kinase
5	gi 30261021	91,361	141	32	19	S-layer protein EA1
6	gi 30258721	87,226	92	17	10	5-Methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
7	gi 30264041	49,452	91	24	9	Dihydrolipoamide dehydrogenase (E3)
8	gi 30261304	70,361	60	16	7	Oligoendopeptidase F
9	gi 30265362	59,749	71	19	7	CTP synthase
10	gi 30264385	65,764	167	21	21	Chaperone protein dnaK
11	gi 30263628	72,408	103	23	14	Transketolase
12	gi 30260443	57,430	175	50	25	Chaperonin, 60 kDa
13	gi 30260481	56,224	172	41	16	Delta-1-pyrroline-5-carboxylate dehydrogenase, putative
14	gi 30260755	61,006	175	31	15	Neutral protease
15	gi 30264541	47,212	97	36	13	Trigger factor
16	gi 30264163	46,223	54	25	9	Pyrimidine-nucleoside phosphorylase
17	gi 30265328	51,192	153	49	22	ATP synthase F1, beta subunit
18	gi 30264042	44,900	72	36	12	Dihydrolipoamide acetyltransferase (E2)
19	gi 30264939	50,344	56	32	9	Phosphoglucose isomerase
20	gi 30260491	52,294	120	39	13	Glutamyl-tRNA(Gln) amidotransferase, A subunit
21	gi 30263502	53,742	113	44	16	Aldehyde dehydrogenase
22	gi 30265485	47,422	83	20	6	Adenylosuccinate synthetase
23	gi 30265161	46,417	188	41	14	Enolase
24	gi 30260328	34,933	68	21	7	DNA-directed RNA polymerase, alpha subunit
25	gi 30253620	42,937	168	61	20	Translation elongation factor Tu
26	gi 30264043	35,228	150	56	13	Pyruvate dehydrogenase complex E1 component, beta subunit
27	gi 30264240	39,850	155	35	13	Leucine dehydrogenase
28	gi 30263830	32,434	57	21	6	Translation elongation factor Ts
29	gi 30260428	43,660	152	30	14	Alanine racemase
30	gi 30265339	45,109	61	11	4	Serine hydroxymethyltransferase
31	gi 30262916	40,023	139	45	17	Chorismate mutase
32	gi 30265022	29,057	60	27	9	ABC transporter, ATP-binding protein
33	gi 30264236	35,790	65	31	9	3-Methyl-2-oxobutanoate dehydrogenase, beta subunit
34	gi 30265359	30,672	95	37	10	Fructose-bisphosphate aldolase, class II
35	gi 30263337	24,006	79	37	7	Transaldolase, putative
36	gi 30260259	32,918	170	65	26	Cysteine synthase A
37	gi 30261955	34,786	81	26	11	L-Lactate dehydrogenase
38	gi 30263838	31,211	63	33	8	Succinyl-CoA synthase, alpha subunit
39	gi 30265165	35,824	117	43	11	Glyceraldehyde 3-phosphate dehydrogenase
40	gi 30264044	41,440	78	21	8	Pyruvate dehydrogenase (E1)
41	gi 30264763	22,323	52	20	4	tRNA binding domain protein, putative
42	gi 30264588	34,340	83	46	13	Electron transfer flavoprotein, alpha subunit
43	gi 30265392	32,389	95	39	12	Agmatinase, putative
44	gi 30264444	25,253	70	54	11	MTA
45	gi 30265465	24,021	120	48	9	Superoxide dismutase, Mn
46	gi 30263350	22,755	150	52	12	Hypothetical protein BA3444
47	gi 30264918	29,691	73	36	10	Naphthoate synthase
48	gi 30261145	31,405	63	32	6	Hypothetical protein BA1021
49	gi 30265367	31,324	61	14	5	3-Hydroxyacyl-CoA dehydrogenase
50	gi 30264875	25,288	58	47	9	Hypothetical protein BA5061
51	gi 30260567	21,101	95	50	8	Tellurium resistance protein
52	gi 30264449	17,437	74	56	6	Transcription elongation factor GreA
53	gi 30261332	17,330	68	56	5	Hypothetical protein BA1237
54	gi 30260515	20,719	139	68	15	Alkyl hydroperoxide reductase, subunit C
55	gi 30260290	18,036	149	66	14	Ribosomal protein L10
56	gi 30263075	18,449	150	52	12	Hypothetical protein BA3129
57	gi 30263828	20,679	109	51	8	Ribosome recycling factor
58	gi 30264160	24,611	71	50	6	Hypothetical protein BA4304
59	gi 30262026	30,100	59	30	6	NH_3-dependent NAD^+ synthetase
60	gi 30263790	9,338	82	78	5	Hypothetical protein BA3920
61	gi 30265409	34,726	70	20	6	Phosphate acetyltransferase
62	gi 30261415	20,412	215	88	20	PhaP protein
63	gi 30265163	26,437	92	42	12	Triosephosphate isomerase
64	gi 30260245	19,691	76	35	8	Stage V sporulation protein T
65	gi 30260944	21,649	95	44	9	$CotJ_C$ protein

Continued on following page

TABLE 1—*Continued*

Spot no.	Accession no.	Theoretical mass (Da)	Mowse score	% Coverage	No. of peptides matching	Protein identification
66	gi 30265393	31,142	130	33	11	Spermidine synthase
67	gi 30265176	21,390	182	53	19	ATP-dependent Clp protease, proteolytic subunit ClpP
68	gi 30264164	29,533	83	28	10	Purine nucleoside phosphorylase
69	gi 30263352	31,828	82	22	7	Oxidoreductase, aldo
70	gi 30263832	28,756	84	25	8	Transcriptional regulator CodY

[a] The spot number identifies the corresponding protein spot on the 2-DE gel as shown in Fig. 1.

Proteins on 2-D gels were transferred to polyvinylidene difluoride (PVDF) membranes using Towbin buffer (0.025 M Trizma base in 0.192 M glycine) with 20% methanol at 100 V for 30 min. After transfer, the PVDF membrane was washed twice for 5 min each in 0.01% Tween 20 in phosphate-buffered saline (PBST) and blocked with 0.2% I-Block (Tropix) for 1 h. To identify immunogenic proteins, the PVDF membranes were washed twice with PBST for 5 min and then probed for one h with either a 1:1,000 dilution of human sera, a 1:500 dilution of goat sera, or a 1:2,000 dilution of rabbit sera. The PVDF membrane was washed twice with PBST and incubated with a 1:5,000 dilution of appropriate secondary antibody. Chemiluminescent signals were visualized using the SuperSignal West Femto Maximum Sensitivity Substrate kit (Pierce Biotechnology). Corresponding antisera from uninfected humans and animals were used as controls. Three replicate blots for each treatment were used for computer analysis using the Phoretix 2D Expression software. All immunogenic proteins that appeared in both control and treated blots were excluded from the list of immunogenic candidate proteins. Precision protein standards plugs (Bio-Rad) were included during electrophoresis as molecular weight standards. Streptactin-horseradish peroxidase conjugate (Bio-Rad) was used as a substrate for visualizing protein markers on 2-D Western blots.

In-gel trypsin digestion and MALDI-TOF MS. Protein spots were picked, washed, and trypsin digested from 2-DE gels according to the manufacturer's instructions using the Xcise robotic workstation (Proteome Systems). Briefly, gel plugs were washed with 50 mM ammonium bicarbonate and 50% acetonitrile, dried, and treated with 1.6 μg/ml of trypsin in 50 mM ammonium bicarbonate at 37°C overnight. Tryptic peptides were applied to a MALDI-TOF MS plate in a solution of 10 mg/ml α-cyano-4-hydroxycinnamic acid in 0.1% trifluoroacetic acid and 50% acetonitrile. MS spectra (100 profiles per spectrum) were obtained using an Axima-CFR Plus MS (Shimadzu Biotech) in a positive ion reflectron mode with a source voltage of 25,000 V and laser intensity of 55%. Peptide mass fingerprints were analyzed and searched against the theoretical spectra of *B. anthracis* strain Ames, *B. cereus* ATCC 14579, or *B. thuringiensis* serovar Konkukian strain 97-27 using the Mascot Daemon software package (Matrix Science). The search parameters were as follows: maximum of one missed cleavage by trypsin, fixed modification of oxidized methionine, charged state of +1, and mass tolerance of ±0.25 Da. With use of these parameters and searching only the respective *Bacillus* sp. database, the probability-based Mowse scores greater than 48 are significant ($P < 0.05$). All MALDI-TOF MS identifications were run at least in duplicate.

RESULTS

Proteomic profiles of *B. cereus* group spores. The 2-DE comparative analysis of the spore proteomes from four representative members of the *B. cereus* group including the avirulent *B. anthracis* strain RA3R, the *B. cereus* avirulent strain 14579, the *B. thuringiensis* virulent strain 97-27, and the *B. thuringiensis* avirulent strain 10972 is presented in Fig. 1 (see also Fig. S1, S2, and S3 in the supplemental material). A total of 1,217 protein spots were detected for all species after computer analysis of SyproRuby-stained average gels, and they were categorized into 245 for *B. anthracis*, 297 for *B. cereus*, 390 for *B. thuringiensis* strain 97-27, and 285 for *B. thuringiensis* strain 10972. Peptide mass fingerprinting resulted in the identification of 458 protein spots (46%) encoded by 232 open reading frames (ORFs) (Fig. 1; Table 1) (see Fig. S1, S2, and S3 and Tables S1, S2, and S3 in the supplemental material). Other spots were not picked because they were either very faint with low molecular weight or not very well separated at pHs 4 to 7. Computer analysis revealed that the spores of each *Bacillus* species can be distinguished from each other by the overall 2-DE protein patterns (Fig. 1) (see Fig. S1, S2, and S3 in the supplemental material).

A number of *Bacillus* spore proteins exist as charge variants with similar molecular masses but differing pIs. Each of these proteins appeared as a train of spots on 2-D gels and was noted for all the spore proteomes of *B. anthracis*, *B. cereus*, and *B. thuringiensis*. In *B. anthracis*, the most notable ones include elongation factor G, 60-kDa chaperonin, dihydrolipoamide dehydrogenase, elongation factor Tu (EF-Tu), components of the pyruvate dehydrogenase (PDH) complex, and enolase (Fig. 1). In *B. cereus* spores, some of the charge variants include acyl coenzyme A dehydrogenase, EF-Tu, and aldehyde dehydrogenase (see Fig. S2 in the supplemental material). In *B. thuringiensis*, the most prominent ones include EF-Tu, enolase, components of the PDH complex, 60-kDa chaperonin, alkylhydroperoxide reductase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phaP protein, camelysin, and Δ-1-pyrroline-5-carboxylate dehydrogenase (see Fig. S3 and S4 in the supplemental material).

Cellular functions. A comparative graphical representation of expressed ORFs categorized into major cellular functions in *B. anthracis*, *B. cereus*, and *B. thuringiensis* strains 97-27 and 10972 is shown in Fig. 2 A to D. For all of the *Bacillus* spores investigated, a majority of the identified proteins are involved in energy production, carbohydrate transport and metabolism, amino acid transport and metabolism, posttranslational modifications, and translation (Fig. 2 A to D). In *B. cereus* and *B. thuringiensis* strain 10792, a number of identified proteins are involved in lipid transport and metabolism (Fig. 2B and D). Since a majority of the spore proteins have metabolic functions that would typically place them in the cytoplasm, most of these proteins may have catalytic and regulatory functions associated with sporulation and germination.

Biomarker proteins. While there are a number of protein spots that are common among all the species tested, there are multiple spots unique to each species, i.e., 21 for *B. anthracis*, 58 for *B. cereus*, and 113 for *B. thuringiensis* (see Fig. S4A in the supplemental material). The term unique spots is based on experimentally determined location (pI and molecular weight coordinates) of these proteins on 2-D gels. A number of these spots are not encoded by unique ORFs. However, some of the unique *B. anthracis* spots include trigger factor, oxidoreductase, phaP protein, and hypothetical protein BA3920. It should be pointed out that despite a thorough spot picking for each

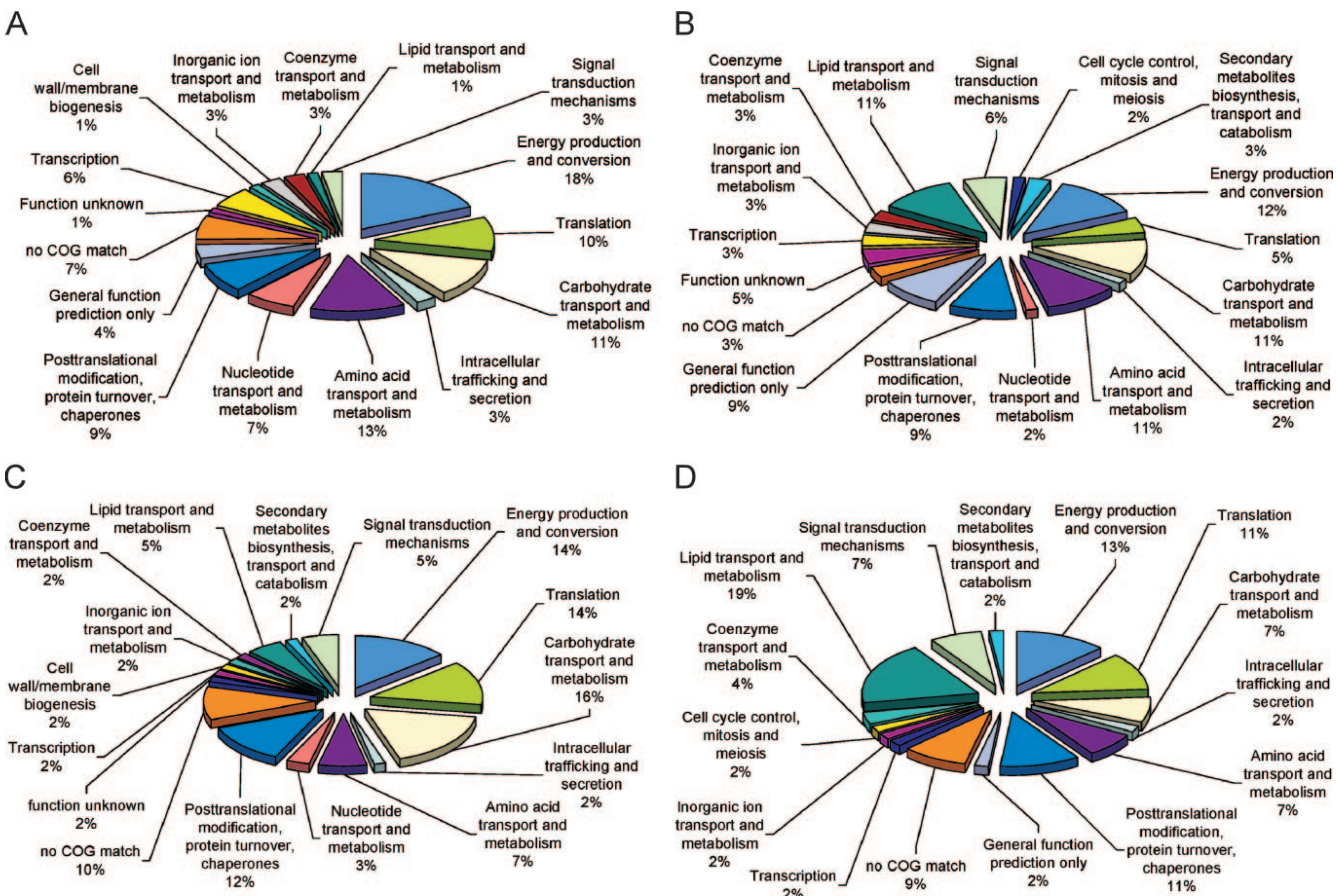

FIG. 2. Graphical representation of expressed ORFs categorized into major cellular functions for *B. anthracis* RA3R (A), *B. cereus* 14579 (B), *B. thuringiensis* 97-27 (C), and *B. thuringiensis* 10972 (D).

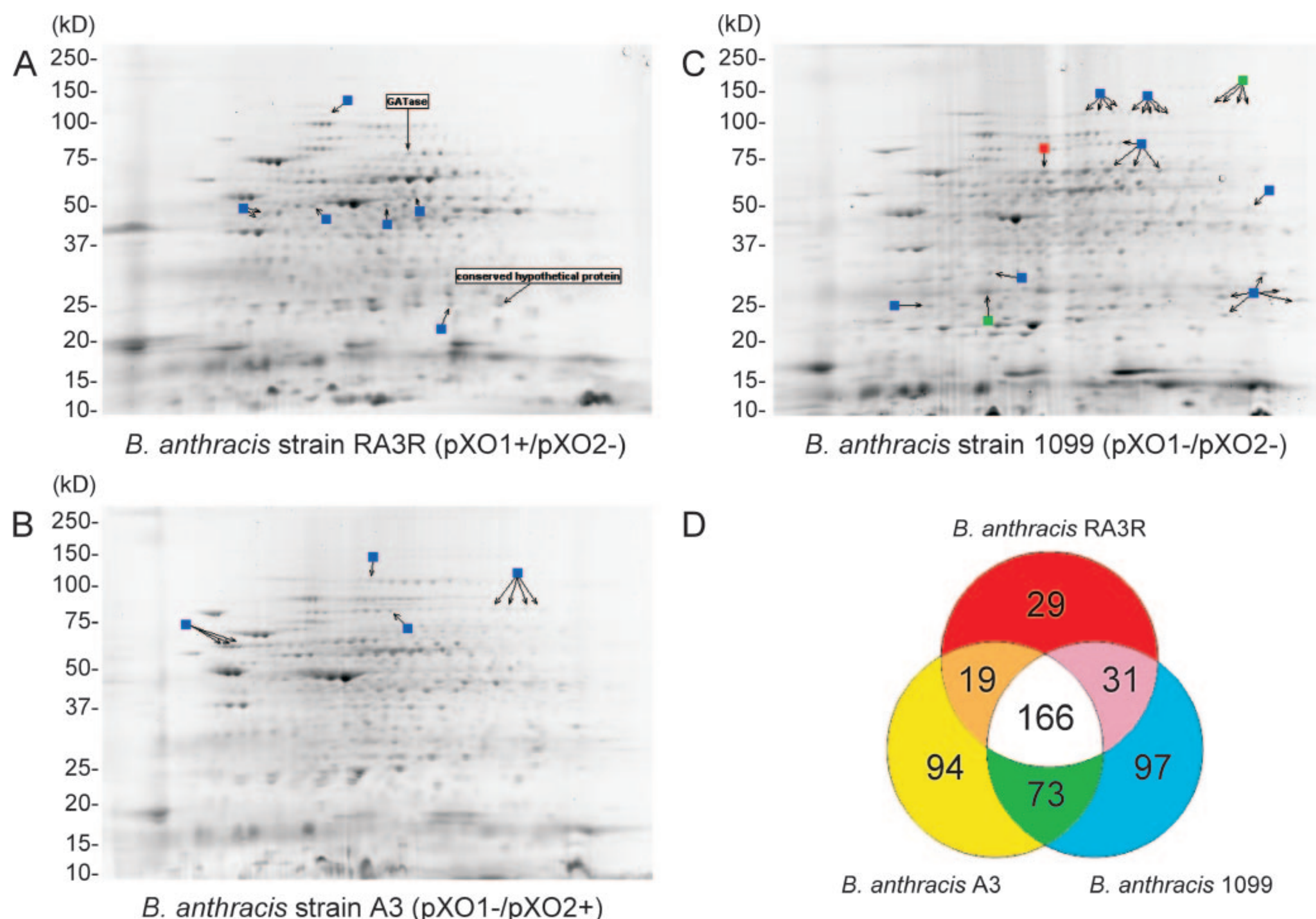

FIG. 3. Spore proteomes of avirulent *B. anthracis* toxigenic and nonencapsulated strain RA3R (pXO1$^+$ pXO2$^-$) (A), nontoxigenic and encapsulated strain A3 (pXO1$^-$ pXO2$^+$) (B), and the totally cured nontoxigenic and nonencapsulated strain 1099 (pXO1$^-$ pXO2$^-$) (C). A Venn diagram illustrating the commonly shared and unique proteins among the three strains is in D. In A to C, blue tags denote protein spots not present in RA3R but present in A3 or 1099; red tags denote protein spots present in 1099 but absent in RA3R or A3; and green tags denote protein spots absent in RA3R and A3.

species, only a few of the unique spots were identified by peptide mass fingerprinting, since a majority of them are not highly expressed and can be deciphered only by computer analysis. Functional identification of all these spots is essential for their value in species classification. Additionally, a total of 196 unique protein spots distinguished the virulent *B. thuringiensis* strain 97-27 from the avirulent strain 10972, which has 91 unique spots (see Fig. S4B in the supplemental material). Overall, the resulting biomarker spots can be used for quick identification of each species using 2-D polyacrylamide gel electrophoresis or for immunocapture assays.

To determine if *B. anthracis* spore protein profiles differ depending on the bacterial plasmid content, strains RA3R (pXO1$^+$ pXO2$^-$), A3 (pXO1$^-$ pXO2$^+$), and 1099 (pXO1$^-$ pXO2$^-$) were examined (Fig. 3A to C). A total of 166 common protein spots were found among the three strains, whereas 29, 94, and 97 protein spots distinguished strains RA3R, A3, and 1099 from each other, respectively (Fig. 3D). The difference in 2-DE spore proteomic profiles may be due not only to plasmid-encoded proteins but also to the altered expression of chromosomally encoded genes that are affected by either pXO1 or pXO2 plasmids or both plasmids.

Spore immunome. Since the endpoint users of a next-generation anthrax vaccine would be humans, proteins specifically immunoreactive to the human population would be preferable as vaccine candidates over those specifically immunoreactive to laboratory-infected animals. A total of 15 immunoreactive proteins were identified in *B. anthracis* strain RA3R (Fig. 4; Table 2). Notably, the most immunodominant antigens include four charge variants of GAPDH (spot 11), three charge variants of EF-Tu (spot 16), dihydrolipoamide acetyltransferase (spot 8), three charge variants of enolase (spot 10), two charge variants of Δ-1-pyrroline-5-carboxylate dehydrogenase (spot 7), alanine racemase (spot 1), the CotJ$_C$ protein (spot 6), and three charge variants of elongation factor G (spot 15) (Fig. 4). Of the 15 antigens listed in Table 2, enolase and 60-kDa chaperonin have been previously identified as components of Anthrax Vaccine Adsorbed (AVA) (39). Using the same human antisera, 7, 14, and 7 immunogenic proteins were identified in *B. cereus* and in *B. thuringiensis* strains 97-27 and 10792, respectively (Table 2).

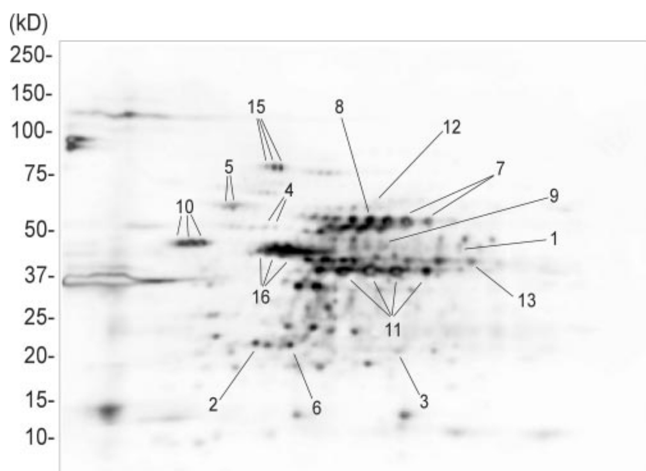

FIG. 4. Immunoblot at pH 4 to 7 of *B. anthracis* strain RA3R spore proteome. The blot was probed with human antisera from patients infected with cutaneous anthrax. The spot numbers correspond to the immunogenic proteins listed in Table 2.

Computer-assisted comparative analysis of Western blots from *B. anthracis*, *B. cereus*, and *B. thuringiensis* indicates that alanine racemase and neutral protease were uniquely immunogenic to *B. anthracis* (Table 2). Overall, the number of immunogenic proteins identified using experimentally infected goat and rabbit antisera (6 and 4, respectively) was lower than those detected using human antisera (data not shown). Two additional immunoreactive proteins, trigger factor (goat antisera) and aldehyde dehydrogenase (goat and rabbit antisera), were detected using animal antisera. This information may be invaluable for the design of veterinary vaccines. The fact that the other 13 *B. anthracis* immunogenic proteins are not unique does not exclude them from being used in a vaccine. In fact, these proteins may be used in a multiagent vaccine that protects against other members of the *B. cereus* group. Furthermore, inclusion of one or more of these newly identified immunogenic proteins in a PA-based vaccine may serve to enhance the overall immunological response and provide a greater level of protection over several life stages of *B. anthracis*.

DISCUSSION

There are numerous studies suggesting that the higher protective efficacy of the spore-based vaccine is due to the contribution of unidentified spore antigens. One study demonstrated that a single subcutaneous administration of 5×10^7 spores from an attenuated *B. anthracis* vaccine strain conferred complete protection to guinea pigs against a lethal challenge of wild-type anthrax spores compared to the partial protection with vaccines administered as vegetative cells (5). Another study has also shown that the administration of a combination vaccine composed of purified PA with 10^8 formaldehyde-inactivated spores of *B. anthracis* with defective lethal factor and edema factor completely protected both guinea pigs and mice from a lethal challenge with wild-type anthrax spores, in contrast to the partial protection conferred by the purified PA-based vaccine alone (4). Using immune sera from monkeys vaccinated with AVA, Welkos and Friedlander (38) have also demonstrated that unidentified anthrax spore surface antigens might be targets of AVA-induced protective immunity. The ability of monkey immune sera to enhance spore phagocytosis by murine peritoneal macrophages was in part due to the humoral immune response against the unidentified spore antigens. Based on these studies, we characterized the unidentified spore antigens of *B. anthracis* and other members of the *B. cereus group* using immunoproteomics approaches. The protein extraction protocol that we used is a modification of the method of Sylvestre et al. (34) for the extraction of the spore's exosporium. Because our extraction protocol was not validated in terms of which spore subproteome is maximally being extracted, we referred to the extracted proteins as "spore proteins" with no specific reference to the exosporium. However, it is most likely that this preparation is highly enriched for exosporial proteins.

Recently, Kudva et al. (20) identified 69 clones by immunological screening of a *B. anthracis* Sterne ($pXO2^+$ $pXO1^-$) limited expression library of putative spore surface proteins with pooled sera from human adults immunized with AVA. Of the 69 immunoreactive spore-associated proteins that Kudva et al. (20) have reported, two immunogenic proteins, i.e., alanine racemase and enolase, were found in common with this study (Table 2). Additionally, alkyl hydroperoxide reductase, subunit C, has been previously reported to be immunogenic in the *B. anthracis* membrane fraction (2). To our knowledge, the other 12 immunogenic proteins listed in Table 2 are novel candidate proteins for developing an improved anthrax vaccine. An immunodominant *B. anthracis* exosporium protein identified by Steichen et al. (33) as BclA (*Bacillus* collagen-like protein) was not identified by mass spectrometry in this or in a similar study

TABLE 2. Identified immunogenic proteins from members of the *B. cereus* group[a]

Protein	Presence in strain			
	Ba RA3R	Bc 14579	Bt 9727	Bt 10792
1. Alanine racemase	+			
2. Alkyl hydroperoxide reductase, subunit C	+	+	+	+
3. ATP-dependent Clp protease	+	+	+	+
4. ATP synthase F1, beta subunit	+	+	+	+
5. Chaperonin, 60 kDa	+		+	
6. $CotJ_c$ protein	+		+	
7. Δ-1-pyrroline-5-carboxylate dehydrogenase	+	+	+	+
8. Dihydrolipoamide acetyltransferase (E2)	+		+	
9. Dihydrolipoamide dehydrogenase (E3)	+		+	
10. Enolase	+	+	+	+
11. Glyceraldehyde-3-phosphate dehydrogenase	+	+	+	
12. Neutral protease	+			
13. Pyruvate dehydrogenase (E1)	+		+	
14. Ribosomal protein L7/L12			+	+
15. Translation EF-G	+		+	
16. Translation EF-Tu	+	+	+	+

[a] 2-D Western blots were probed using antisera from human patients infected with cutaneous anthrax. Ba, *B. anthracis*; Bc, *B. cereus*; Bt, *B. thuringiensis*. The number preceding each protein corresponds to the spot number in Fig. 4.

(24) because this protein does not have any trypsin cleavage sites.

A number of the immunogenic spore proteins found in this study are associated with generation of metabolic energy, bacterial survival, or enzymatic processes relevant to germination. GAPDH, Clp protease, enolase, ATP synthase, and the PDH complex have associations with ATP generation. GAPDH is particularly interesting because it is currently being tested in a number of systems for development of vaccines against *Edwardsiella tarda* (17, 25), *Streptococcus pneumoniae* (16, 23), and *Onchocerca volvulus* (10). All of these studies suggest that the use of GAPDH alone or as a component of an anthrax vaccine formulation may similarly provide protection against *B. anthracis* infection. Clp protease is an ATP-dependent enzyme consisting of an ATPase specificity factor, the heat shock protein 100/clp family member ClpC, and the proteolytic subunit ClpP. Clp proteases are fundamental in many pathogenic bacteria for stress tolerance and virulence (15). The glycolytic enzyme enolase was also found to be immunogenic. A recent study has reported that enolase induced in vitro neutralizing antibodies against *Chlamydia pneumoniae* cells and inhibited their dissemination with a hamster model, indicating its value as a vaccine component (11). The PDH complex is composed of three enzymes, i.e., PDH (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3), each of which was found to be immunogenic (Table 2). The PDH complex converts pyruvate to acetyl coenzyme A, which is subsequently used in the tricarboxylic acid cycle to generate NADH, ATP, and reduced flavin adenine dinucleotide (7). The PDH complex is known to be highly immunogenic in other bacterial species, such as *Neisseria meningitidis* (1), *Mycoplasma capricolum* (40), and *Mycoplasma hyopneumoniae* (28). Recently, PDH has been tested as a DNA vaccine against *Mycoplasma mycoides* subsp. *mycoides*, the causal agent of contagious bovine pleuropneumonia (27). The study showed that significant anti-*M. mycoides* subsp. *mycoides* responses were observed in mice vaccinated with clones containing the full-length genes of the PDH complex.

The identification of new immunoreactive proteins from the spores of *B. anthracis*, *B. cereus*, and *B. thuringiensis* adds to the list of potential targets for developing more-specific, safer, and highly efficacious vaccines. Since several of these immunoreactive proteins are common among members of the *B. cereus* group, it is possible to develop not only a potent anthrax vaccine but also a multiagent and multivalent vaccine against *B. cereus* and *B. thuringiensis*, where some strains are pathogenic to humans. The same immunoproteomics approach can be used for other infectious agents, including viruses, where no vaccine is currently available for treatment. One revolutionary approach is to incorporate the genes encoding these proteins into bacterial ghosts (BG), a nonliving and nonpathogenic envelope of gram-negative bacterium that is formed by the expression of the cloned bacteriophage ΦX174 *E* gene (26, 36). The potential of cloning and expressing genes encoding immunogenic proteins into BG has been discussed in a recent review (18). A new era in vaccine development is emerging that utilizes the advances in immunoproteomics and the BG platform technology for the design and creation of potent biodefense vaccines.

Comparative analysis of the spore proteomes from three members of the *B. cereus* group has also shown that it is possible to distinguish *B. anthracis* from *B. cereus* and *B. thuringiensis* based on their 2-DE protein signatures and the presence of spots unique to each species. This is important for differential detection and identification of each species and may lead to the development of DNA- or immuno-based detection platforms. The same approach has been used to discriminate various strains of *B. anthracis* that differ in their plasmid content, as well as the virulent from avirulent strains of *B. thuringiensis*. These data are valuable for a systems biology approach for studying the complete biology of these pathogens and making better predictions of their virulence.

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