Identification of *Bacillus anthracis* Spore Component Antigens Conserved across Diverse *Bacillus cereus sensu lato* Strains*[S]

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We sought to identify proteins in the *Bacillus anthracis* spore, conserved in other strains of the closely related *Bacillus cereus* group, that elicit an immune response in mammals. Two high throughput approaches were used. First, an *in silico* screening identified 200 conserved putative *B. anthracis* spore components. A total of 192 of those candidate genes were expressed and purified *in vitro*, 75 of which reacted with the rabbit immune sera generated against *B. anthracis* spores. The second approach was to screen for cross-reacting antigens in the spore proteome of 10 diverse *B. cereus* group strains. Two-dimensional electrophoresis resolved more than 200 protein spots in each spore preparation. About 72% of the protein spots were found in all the strains. 18 of these conserved proteins reacted against anti-*B. anthracis* spore rabbit immune sera, two of which (alanine racemase, Dal-1 and the methionine transporter, MetN) overlapped the set of proteins identified using the *in silico* screen. A conserved repeat domain protein (Crd) was the most immunoreactive protein found broadly across *B. cereus sensu lato* strains. We have established an approach for finding conserved targets across a species using population genomics and proteomics. The results of these screens suggest the possibility of a multiepitope antigen for broad host range diagnostics or therapeutics against *Bacillus* spore infection. *Molecular & Cellular Proteomics 8:1174–1191, 2009.*

The anthrax causing bacterium *Bacillus anthracis* is a member of the *Bacillus cereus sensu lato* (*s.l.*)[1] group, a term given to the polyphyletic species consisting of *Bacillus thuringiensis*, *Bacillus cereus*, *Bacillus mycoides*, *Bacillus weihenstephanensis*, and *Bacillus pseudomycoides* (1). Genomics studies of *B. cereus s.l.* strains have shown a similar chromosomal gene composition within this group (2–7). Many phenotypes that distinguish *B. cereus s.l.* members, such as crystalline toxin production (8), emesis in humans (9), and anthrax virulence (10), are encoded by genes on large plasmids. Experimental conjugative transfer of plasmids between *B. cereus s.l.* strains has been demonstrated *in vitro*, in complex media, and in vector species (11–13). Therefore there is a concern about transfer of virulence genes between genetic backgrounds creating new pathogen lineages. In this regard, there is an emerging evidence of natural dissemination of the pXO1 and pXO2 plasmids that encode the anthrax lethal toxin and capsule, respectively. For example, *B. cereus* G9241 carries a pXO1 plasmid and lethal toxin genes almost identical to those in *B. anthracis* (6), and a *B. cereus* strain, which causes anthrax-like illness in African great apes, apparently contains both pXO1 and pXO2 plasmids (14).

The infectious agent of most if not all human *B. cereus s.l.* diseases is the spore. The spore is a dormant, environmentally resistant structure that persists in nutrient- or water-limiting conditions. Anthrax infection occurs after introduction of the *B. anthracis* spore into a skin abrasion or via inhalation or ingestion (10). The spore germinates inside host cells, and the resulting vegetative bacteria express toxins and capsules that elicit an immune response (10, 15, 16). Formation of the *B. cereus* spore involves asymmetric cell division during which a copy of the genome is partitioned into each of the sister cells. The smaller cell (prespore) develops into mature endospore, and the larger cell (mother cell) contributes to the differentiation process but undergoes autolysis following its completion to release the endospore into the surrounding medium. Synthesis of cortex, coat, and exosporium are a function mainly of the mother cell. The cortex and coat layers are in close proximity to one another, whereas the exosporium

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Received, August 27, 2008, and in revised form, February 6, 2009
Published, MCP Papers in Press, February 9, 2009, DOI 10.1074/mcp.M800403-MCP200

[1] The abbreviations used are: *s.l.*, *sensu lato* (translation, "in the broad sense"); ATCC, American Type Culture Collection; BGSC, *Bacillus* Genetic Stock Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; IS, interspace (distance between endospore and exosporium); NCBI, National Center for Biotechnology Information; RAESS, rabbit anti-exosporium sera; RAGSS, rabbit anti-GerH whole spore sera; RANS, rabbit anti-naïve sera; RAWSS, rabbit anti-whole spore (ungerminated) sera; TAP, Transcriptionally Active PCR; TEM, transmission electron microscopy; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; BLAST, basic local alignment search tool; RTS, Rapid Translational System; 2D, two-dimensional; ABC, ATP-binding cassette; AMU, atomic mass unit; PA, protective antigen.

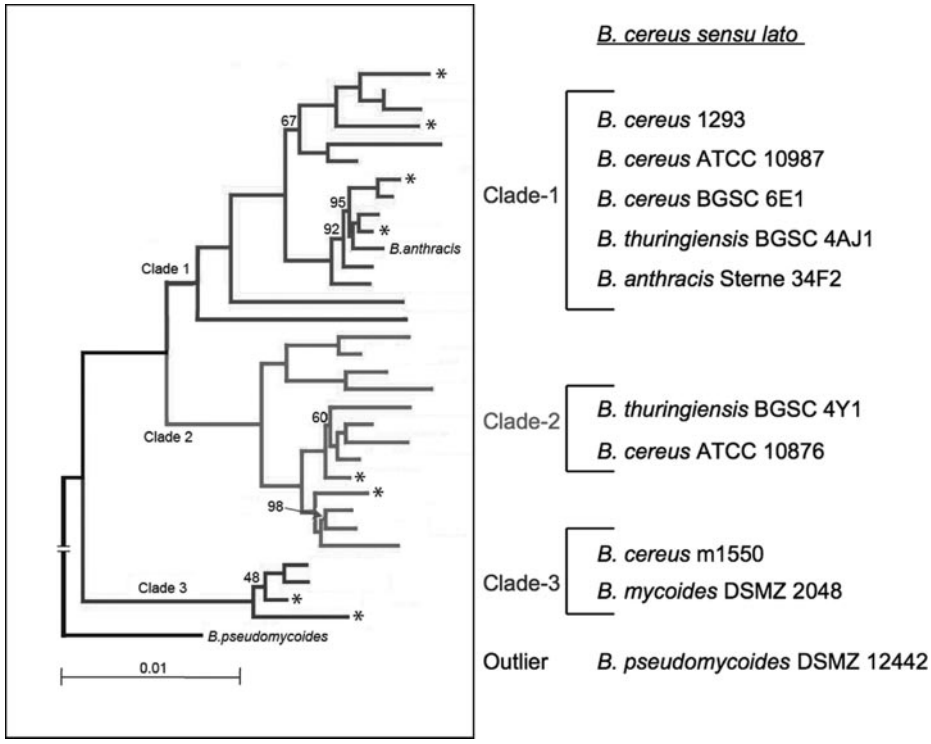

FIG. 1. **Phylogeny of *B. cereus s.l.* genomes.** A neighbor-joining tree was created from a concatenation of 373 conserved, single copy proteins from 33 *B. cereus* group genomes (67). Each branch has 100% recovery in 100 bootstrap replicates unless indicated on the tree. The branches of the strains selected for this study are marked with *asterisks* and correspond to the strains listed from *top* to *bottom*.

tends to appear as an irregularly shaped, loosely attached, balloon-like layer (17–20). The coat and the exosporium contribute to the remarkable resistance of spores to extreme physical and chemical stresses including the exposure to extraterrestrial conditions (21, 22). Recent work on the structure, composition, assembly, and function of the spore coat and exosporium of pathogenic organisms like *B. anthracis* and *B. cereus* have highlighted the crucial link that exists between the origin of these layers (19, 23). There are differences in the appearance and thickness of the coat layers among the spores of various strains and species. In some *B. thuringiensis* strains, the inner coat is laminated but consists of a patchwork of striated packets, appearing either stacked or comblike, and the outer coat is granular (24), whereas in *B. anthracis* and other *B. cereus s.l.* isolates the coat appears compact (25–27). The coat layers comprise about 30% of the total proteins present in the spore (19, 28). Intraspecies variation in the structure and composition of the spore surface layers may reflect the environmental conditions under which these spores are formed (29–31).

Because the spore is crucial to infection and persistence of *B. anthracis* and its close relatives, we undertook an investigation of its protein profile variability across the *B. cereus s.l.* group. Our goal in this study was to identify conserved antigenic spore proteins that may be transitioned in the future as candidates for immunodiagnostics, therapeutics, or vaccines. We used two high throughput approaches: genome-based bioinformatics analysis and comparative proteomics analysis of spores of *B. cereus s.l.* to select conserved targets. Our analysis revealed a list of conserved spore proteins within *B. cereus* but relatively few cross-reacting antigens. Two of these spore conserved antigens (Crd and MetN) have not been described previously for *B. anthracis.*

EXPERIMENTAL PROCEDURES

Bacterial Cultures—For comparative proteomics analysis, 10 strains from the *B. cereus* group were selected: *B. anthracis* Sterne 34F2 (a pXO2$^-$ laboratory strain); *B. thuringiensis* BGSC 4AJ1 and BGSC 4Y1; five strains of *B. cereus*, ATCC 10987, ATCC 10876, BGSC 6E1, m1293 (Oslo, Norway), and m1550; *B. mycoides* DSMZ 2048; and *B. pseudomycoides* DSMZ 12442 (sources of the strains are shown in Supplement 1). Strains for analysis were selected on the basis that they represented distinct branches on a phylogeny of the *B. cereus* group based on a whole genome sequencing study (Fig. 1) (67). Whole genome shotgun sequences of these strains (except *B. anthracis* Sterne 34F2 (32) and *B. cereus* ATCC 10987 (4)) were obtained using 454 pyrosequencing technology to an average coverage of ~25 reads per base (33) as part of a study on genome variation in this group (67).

Sequence Accession Numbers—Submission of the whole genome shotgun data to GenBank™ is currently in process. The NCBI Genome Project IDs are given in parentheses: *B. thuringiensis*, BGSC 4AJ1 (29709) and BGSC 4Y1 (29713); four strains of *B. cereus*, ATCC 10876 (29671), BGSC 6E1 (29651), m1293 (29657), and m1550 (29649); *B. mycoides* DSMZ 2048 (29701); and *B. pseudomycoides* DSMZ 12442 (29707).

Bacterial Spore Preparation—The growth curves of each of the *Bacillus* strains in brain-heart infusion broth were determined to estimate the time of late logarithmic phase for each of the strains (Supplement 2). One colony from a blood agar plate was used to inoculate 5 ml of brain-heart infusion broth. At late logarithmic phase (~6 h of shaking at 250 rpm at 37 °C) 1.0 ml was transferred into 50 ml of modified G medium (34) and incubated at 37 °C for about 72 h at 250 rpm to induce sporulation. The end point of sporulation was determined when the proportion of spores observed under a phase-

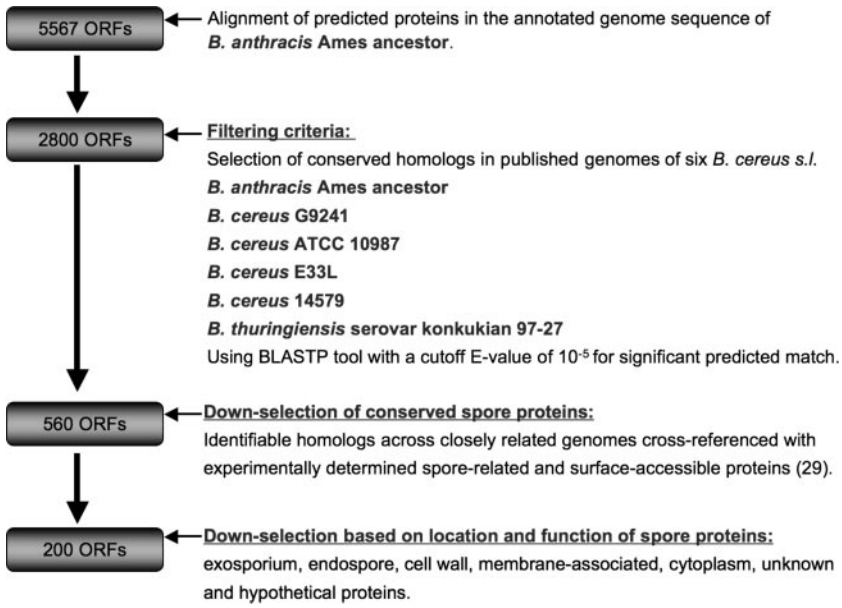

FIG. 2. **Filtering strategy for down-selection of spore-associated conserved ORFs from *B. cereus s.l.***

contrast microscope was more than 20 times that of the vegetative cells. The spore suspensions were heated at 65 °C for 30 min to kill the remaining vegetative cells. The heat-treated spore suspensions were centrifuged at 6000 rpm (4,350 × *g*) at 25 °C for 30 min and washed extensively with cold (4 °C) Milli-Q water. Spore pellets were purified through a 50% renograffin layer (Bracco Diagnostics) (35) prepared in Milli-Q water and centrifuged at 4,350 × *g* for 30 min at 25 °C to remove cell debris. The spore pellets were further washed three times with cold Milli-Q water to remove traces of renograffin. The purified spore suspensions were stored in Milli-Q water at 4 °C in 500-μl aliquots. Spores were quantified microscopically with a hemocytometer.

Transmission Electron Microscopy (TEM) Analysis—*Bacillus* spore samples were prepared for transmission electron microscopy examination as described previously (36) with slight modification. In brief, spores were fixed in 1% osmium tetroxide and 2% potassium permanganate in 0.1 M phosphate buffer for 2 h at room temperature. Following fixation, spores were thoroughly rinsed, enrobed in agar, and trimmed into 1 mm^3. The enrobed material was stained in the block with uranyl acetate, dehydrated by passage through an ethanol series, and embedded in LR White. Sections were cut on a Reichert Jung ultramicrotome Ultracut E, stained with lead citrate, and examined in an FEI Tecnai T12 transmission electron microscope at an accelerating voltage of 80 kV (36). TEM images of the spore samples were examined to verify that (i) whole spore preparations contained both endospores with intact exosporium and (ii) exosporium preparations were not contaminated with endospores (and vice versa).

Extraction of Spore Proteins—The protein extraction method was adapted from several reported protocols (29, 35, 37). Spore suspensions were centrifuged at 13,200 rpm (16,100 × *g*) at 25 °C for 10 min, and the pellets were resuspended in 500 μl of lysis buffer containing ZOOM 2D protein solubilizing buffer-1 (Invitrogen) and protease inhibitor mixture (Roche Applied Science). The samples were pulse-sonicated for 15 s for four times at level 4 with a Model 100 cell dismembrator (Fisher Scientific) followed by addition of 4.0 μl of sample buffer (50 mM $MgCl_2$, 1 unit of DNase I, 3 units of RNase A, and 0.5 M Tris-HCl) and incubation in an ice bath for 10 min to eliminate DNA and RNA contamination. The samples were stored at −80 °C in 10-μl aliquots.

Two-dimensional Gel Electrophoresis Separation of Spore Proteins—The spore protein extracts were mixed with IPG rehydration buffer (8 M urea, 4% (w/v) CHAPS, 0.04 M Tris base, 0.065 M dithiothreitol, and 0.01% (w/v) bromphenol blue) in a final volume of 260 μl. To generate an electrophoretic map of each of the spore preparations 50 μg of protein extract was mixed with IPG rehydration buffer, loaded onto Immobiline™ DryStrip (ReadyStrip™ IPG strips; pH 4–7, 6–11, or 3–10; strip length, 11 cm; Bio-Rad), allowed to rehydrate overnight, and isoelectrofocused to 20,000 V-h in a PROTEAN® IEF cell (Bio-Rad). The following focusing parameters were applied. A rapid advance voltage ramping method was used in which 250 V for 15 min was used for ramping in the first step followed by 8,000 V for 2.5 h in the second step, and finally 20,000 V for 6 h was used for the final focusing step. A hold step was programmed to maintain the voltage at 500 V until the run was stopped. The maximum current limit per gel was set at 50 μA. After focusing was completed, IPG strips were equilibrated with 2% (w/v) dithiothreitol in equilibration base buffer containing 6 M urea, 2% SDS, 20% (v/v) glycerol, 0.375 M Tris-HCl, and 0.001% bromphenol blue for 15 min followed by another equilibration step with 2.5% (w/v) iodoacetamide in equilibration base buffer for 15 min. The second dimension was carried out with Criterion XT precast 4–12% bis-Tris, 11.0-cm slab gels in a Criterion Dodeca multicell system (Bio-Rad) at 4 °C for 1 h at 200 volts in 1× NuPAGE MOPS-SDS buffer (Invitrogen) with 2.5% NuPAGE antioxidant (Invitrogen) in the upper electrode buffer. Gels were fixed in 40% ethanol and 10% acetic acid for 30 min followed by staining with a SilverQuest silver staining kit (Invitrogen). Images were scanned in the ChemiDoc XRS system (Bio-Rad). This imaging equipment is fully integrated to the ProteomeWorks™ system (Bio-Rad) with a high resolution image acquisition interface with the PDQuest software.

Image Analysis—Protein spots were analyzed for differential expression patterns using PDQuest software version 7.2 (Bio-Rad). For quantification, a match set of all the gels was generated, and the spots were normalized to the total quantity of the valid spots in each of the gel images in the match set. After normalization, each spot in the master gel was matched with the same spot in each of the gels as mentioned previously (38, 39). For statistical analysis, a matching summary of all the spots in all the gels was generated.

Protein Spot Identification and Data Analysis—Protein spots of interest were excised from silver-stained gels. In-gel digestion was performed using a modified method of Havlis *et al.* (40). Extracts from the digest were separated from the matrix using C_{18} ZipTips. Eluent from the ZipTips was lyophilized to dryness and suspended in 10 μl

TABLE I

Conserved spore proteins of B. anthracis and closely related strains selected by genome-based in silico analysis showing reaction with rabbit immune sera

Results were derived from subtraction of control serum (unimmunized, 0.005 at A_{405}) from anti-GerH whole spores and anti-exosporium sera. −, <0.1; +, 0.1–0.2; ++, 0.2–0.3; +++, >0.3. PTS, phosphotransferase system.

Genes selected	ORFs	Molecular mass	ELISA with rabbit immune sera	
			Anti-spore	Anti-exosporium
	bp	*kDa*		
Sporulation				
gi47505340; GBAAT34016.1; small, acid-soluble spore protein B	198	7.3	−	++
gi47504727; GBAAT33403.1; stage V sporulation protein AF	1,473	54.0	+	−
gi47504853; GBAAT33529.1; stage III sporulation protein AG	663	24.3	++	+
gi47505104; GBAAT33780.1; forespore-specific protein, putative	627	23.0	+	−
gi47504730; GBAAT33406.1; stage V sporulation protein AD	1,017	37.3	+	+
gi47505076; GBAAT33752.1; prespore-specific transcriptional regulator rsfA, putative	642	23.5	+	−
gi47505119; GBAAT33795.1; spo0B-associated GTP-binding protein	1,287	47.2	+	−
gi47503187; GBAAT31863.1; spore cortex lytic enzyme prepeptide	762	27.9	+	−
gi47501241; GBAAT29917.1; cotJB protein	276	10.1	−	++
Cell division				
gi47500466; GBAAT29142.1; cell division protein FtsH	1,902	69.7	+++	+
gi47504423; GBAAT33099.1; signal recognition particle-docking protein FtsY	990	36.3	+	−
gi47504476; GBAAT33152.1; cell division initiation protein DivIVA	507	18.6	+	−
gi47504487; GBAAT33163.1; cell division protein FtsA	1,302	47.7	++	−
gi47504408; GBAAT33084.1; gid protein	1,305	47.9	−	++
ABC transporter				
gi47500766; GBAAT29442.1; iron compound-binding protein ABC transporter	918	33.7	+++	+
gi47505671; GBAAT34347.1; ABC transporter, ATP-binding protein	786	28.8	−	+
gi47505676; GBAAT34352.1; ABC transporter, ATP-binding protein	1,026	37.6	+	−
gi47500801; GBAAT29477.1; ABC transporter, substrate-binding protein, putative	999	36.6	++	−
gi47551555; GBAAT29896.2; Na/P_i-cotransporter family protein	1,656	60.7	−	+
gi47501472; GBAAT30148.1; ABC transporter, ATP-binding protein EcsA	744	27.3	+	+
gi47501606; GBAAT30282.1; oligopeptide ABC transporter, ATP-binding protein	1,044	38.3	−	++++
gi47501607; GBAAT30283.1; oligopeptide ABC transporter, ATP-binding protein	936	34.3	+	++
gi47501710; GBAAT30386.1; spermidine/putrescine ABC transporter, ATP-binding protein	996	36.5	+	−
gi47501714; GBAAT30390.1; spermidine/putrescine-binding ABC transporter protein	1,038	38.1	+	+
gi47502979; GBAAT31655.1; ABC transporter, ATP-binding protein	669	24.5	+	−
gi47503226; GBAAT31902.1; glycine betaine/L-proline ABC transporter, permease protein	837	30.7	++	+
Hypothetical				
gi47502779; GBAAT31455.1; hypothetical protein	537	19.7	++++	++++
gi47505416; GBAAT34092.1; hypothetical protein	420	15.4	+	−
gi47501650; GBAAT30326.1; hypothetical protein	504	18.5	++	+
gi47501749; GBAAT30425.1; hypothetical protein	342	12.5	+	+
gi47504707; GBAAT33383.1; hypothetical protein	363	13.3	++	+
Conserved hypothetical				
gi47506115; GBAAT34791.1; conserved hypothetical protein	438	16.1	−	+
gi47501825; GBAAT30501.1; conserved hypothetical protein	555	20.4	+	+
gi47502061; GBAAT30737.1; conserved hypothetical protein	249	9.1	+	+
gi47551733; GBAAT31416.2; conserved hypothetical protein	756	27.7	−	+++
gi47551862; GBAAT32537.2; conserved hypothetical protein	1,062	38.9	++++	+++
gi47504112; GBAAT32788.1; conserved domain protein	264	9.7	+	+++
gi47504632; GBAAT33308.1; conserved hypothetical protein	213	7.8	++	−
gi47503999; GBAAT32675.1; conserved hypothetical protein	1,467	53.8	+	−
gi47504358; GBAAT33034.1; conserved hypothetical protein	912	33.4	+	−
gi47504360; GBAAT33036.1; conserved hypothetical protein	249	9.1	++	−
gi47504363; GBAAT33039.1; conserved hypothetical protein	1,275	46.8	+	−
gi47504372; GBAAT33048.1; conserved hypothetical protein	750	27.5	+	−
gi47504390; GBAAT33066.1; conserved hypothetical protein	273	10.0	+	−
gi47501585; GBAAT30261.1; conserved hypothetical protein	198	7.3	−	++

TABLE I—*continued*

Genes selected	ORFs	Molecular mass	ELISA with rabbit immune sera	
			Anti-spore	Anti-exosporium
	bp	*kDa*		
Lipoprotein				
gi47505654; GBAAT34330.1; lipoprotein, putative	576	21.1	+++	+
gi47500656; GBAAT29332.1; lipoprotein, putative	1,014	37.2	++	−
gi47503185; GBAAT31861.1; lipoprotein, putative	597	21.9	+	−
gi47502474; GBAAT31150.1; adhesion lipoprotein	951	34.9	++	−
Spore germination				
gi47500552; GBAAT29228.1; spore germination protein GerD	618	22.7	++	−
gi47502385; GBAAT31061.1; bacterial luciferase family protein	1,056	38.7	+	−
DNA binding				
gi47504294; GBAAT32970.1; DNA-binding protein HU	273	10.0	+	++
gi47500486; GBAAT29162.1; DNA-binding protein, putative	1,074	39.4	+	−
Amino acid binding				
gi47552115; GBAAT34692.2; ATP synthase F_1, γ subunit	861	31.6	−	+
gi47504102; GBAAT32778.1; thioredoxin-like protein	198	7.3	+	+++
gi47504630; GBAAT33306.1; polypeptide deformylase	555	20.4	+	+++
Energy metabolism				
gi47552124; GBAAT35466.1; 4-oxalocrotonate tautomerase	186	6.8	−	+
gi47504281; GBAAT32957.1; PTS system, fructose-specific IIABC component	1,881	69.0	+	−
gi47504349; GBAAT33025.1; pyruvate ferredoxin oxidoreductase, α subunit, putative	1,758	64.5	+	−
Cell wall				
gi47551930; GBAAT33049.2; metallo-β-lactamase family protein	1,671	61.3	+	−
gi47503556; GBAAT32232.1; metallo-β-lactamase family protein	846	31.0	+++	−
gi47500657; GBAAT29333.1; alanine racemase	1,170	42.9	−	++
gi47502504; GBAAT31180.1; chaperone CsaA	330	12.1	−	+
Protein degradation				
gi47504362; GBAAT33038.1; zinc protease, insulinase family	1,287	47.2	+	−
gi47504380; GBAAT33056.1; zinc protease, insulinase family	1,242	45.5	+	−
gi47504406; GBAAT33082.1; ATP-dependent protease hsIV	543	19.9	+	−
gi47504350; GBAAT33026.1; renal dipeptidase family protein	924	33.9	+	−
Cellular process				
gi47504391; GBAAT33067.1; N-utilization substance protein A	1,107	40.6	+	−
gi47504101; GBAAT32777.1; glycosyl hydrolase, family 18	1,293	47.4	+	+
gi47504599; GBAAT33275.1; cytochrome *c* oxidase, subunit I	1,836	67.3	+	−
Translation				
gi47500532; GBAAT29208.1; ribosomal protein L30	183	6.7	−	+
gi47500548; GBAAT29224.1; ribosomal protein S9	393	14.4	++	++
gi47504277; GBAAT32953.1; host factor-I protein	225	8.3	+	++
gi47504383; GBAAT33059.1; ribosomal protein S15	270	9.9	−	+
gi47502550; GBAAT31226.1; ATP-dependent RNA helicase, DEAD/DEAH box family	1,170	42.9	+	−
Control				
Exosporium preparation			++++	++++

of 2% acetonitrile and 0.1% trifluoroacetic acid. Electrospray ionization mass spectrometry was carried out using a ThermoFinnigan LTQ (linear trap quadrupole) mass spectrometer equipped with a surveyor liquid chromatography pump. The samples were injected using a Finnigan microautosampler. About 6.4 μl was injected onto a 75-μm-inner diameter nanospray column. The column had a 15-μm opening and was packed with 10 cm of BioBasic C_{18}. A 120-min gradient was used with a two-component solvent system with 0.1% formic acid as solvent A and 0.1% formic acid in acetonitrile as solvent B. The gradient was as follows: 0–40 min, 2% solvent B; 40–65 min, 60% solvent A and 40% solvent B; 65–70 min, 10% solvent A and 90% solvent B; 70–75 min, 98% solvent A and 2% solvent B; and 82–110 min, 98% solvent A and 2% solvent B. The flow rate was 250 μl/min presplit. The flow rate was 800 nl/min at the tip.

The mass spectrometer was operated in double play mode with one full-scan mass spectrum followed by nine data-dependent MS/MS scans. The minimum signal required for a data-dependent scan was 100. Dynamic exclusion was enabled with a repeat count of 3 and a repeat duration of 30 min. Automatic gain control target values were 3000 for full-scan MS and 1000 for MS/MS. Normalized collision energy for collision-induced dissociation was 35. Capillary voltage was 49 V, and spray voltage was 2.0 kV.

Extract_msn in Bioworks 4.0 software (version 3.3) was used to generate the peak list. The SEQUEST algorithm was used to interpret MS/MS data (41). The report format of the SEQUEST database (a part of Bioworks version 3.3) search lists the proteins in order of probability. The protein probability score is based on the probability that a peptide is a random match to the spectral data. The best peptide probability value was used as the probability value for protein identification. If a protein was identified by four or more unique peptides with SEQUEST scores that met or exceeded the acceptance criteria, the protein was considered to be present in the sample analyzed. The search parameters included up to two missed trypsin cleavages with methionine oxidation as variable modification and no fixed modifica-

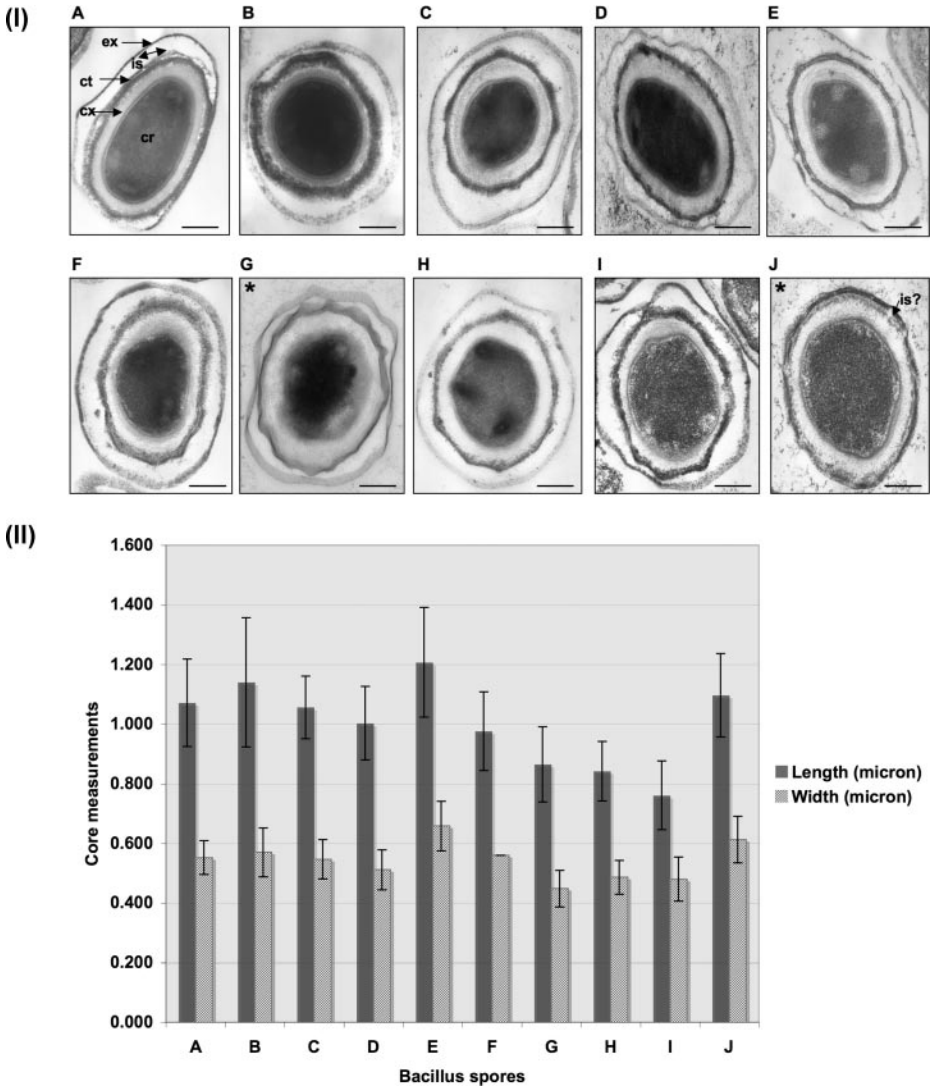

FIG. 3. *Panel I*, TEM showing ultrastructural analysis of *B. anthracis* and closely related strains. *A*, *B. anthracis* Sterne 34F2; *B*, *B. thuringiensis* BGSC 4AJ1; *C*, *B. cereus* m1293; *D*, *B. cereus* BGSC 6E1; *E*, *B. cereus* ATCC 10987; *F*, *B. cereus* ATCC 10876; *G*, *B. thuringiensis* BGSC 4Y1; *H*, *B. cereus* m1550; *I*, *B. mycoides* DSMZ 2048; *J*, *B. pseudomycoides* DSMZ 12442. *ex*, exosporium; *is*, interspace; *ct*, coat; *cx*, cortex; *cr*, core. The *bar* is 100 nm at magnification ×60,000. All the spores were prepared using the modified G medium at 37 °C up to 72 h at 250 rpm and purified using a 50% renograffin gradient. *Panel II*, core dimensions of the spores of closely related *Bacillus* strains. *A–J* show the same strains listed for *panel I*. *Error bars* represent standard deviation in microns.

tions. The mass tolerance for precursor ions and fragment ions were considered as 2 and 1 AMU, respectively. Patterns of measured masses were also matched against theoretical masses of proteins found in the NCBI, Swiss-Prot, and TrEMBL databases accessible in the ExPASy Molecular Biology server. We also searched a subset database created from the NCBI non-redundant database using a search term of "*Bacillus*" proteins and an in-house Biological Defense Research Directorate genome database of predicted proteins from genomes that were sequenced and run through our in-house annotation pipeline. This pipeline consists of a collection of common bioinformatics applications including Glimmer3 (42) for *ab initio* gene prediction and BLAST-based homology search against a reference genome for gene function assignment, in this case *B. anthracis* Ames ancestor strain (NCBI RefSeq accession numbers NC_007530, NC_007322, and NC_007323). Sequence coverage >30% was considered as a positive match. Distinct protein spots in the Western blots that could not be identified by LC-MS (resulting from an insufficient amount of protein in the spot, and/or a clearly resolved spot was not obtained in the silver-stained electrophoretic map) were identified by a bioinformatics approach using the TagIdent tool. The molecular weight and pI of such spots were estimated from the analytical gels and applied to TagIdent with very stringent parameters and specified error margins (pI range = 0.25 and molecular weight range = 10%) to match the proteins from the database (Swiss-Prot/TrEMBL/NCBI) near to the defined range of molecular weight and pI with a p value of <0.05.

In Vitro Transcription and Translation—A total of 200 target ORFs were amplified and expressed using the high throughput cell-free Transcriptionally Active PCR (TAP) fragment system developed by Gene Therapy Systems Inc., San Diego, CA (43). Gene-specific primers for 200 genes were generated using the *B. anthracis* Sterne 34F2 genome sequence. Primers complementary to the 5′- and 3′-ends of the gene of interest were synthesized. The 5′ and 3′ primers were mixed and added to plates containing *Taq* polymerase (Clontech-BD Biosciences) and *B. anthracis* Sterne 34F2 genomic DNA. The PCR was carried out in a thermocycler PCR machine (MWG Biotech, AG) for 30 cycles. Upon completion, PCRs were transferred to a Millipore Montage 96-well cleanup plate and filter-purified using a vacuum manifold. About 10–20 ng of DNA from each PCR from the previous step was used as a template in the second step PCR that also included the DNA fragments containing T7 promoter, His_6 sequences, and T7 terminator sequences. This step generated a DNA fragment that contained the gene of interest with the added 5′- and 3′-TAP universal end sequences. The PCR, subsequent purification, and quantification of the PCR product was carried out as detailed above. Promoter and terminator sequences were added onto the TAP

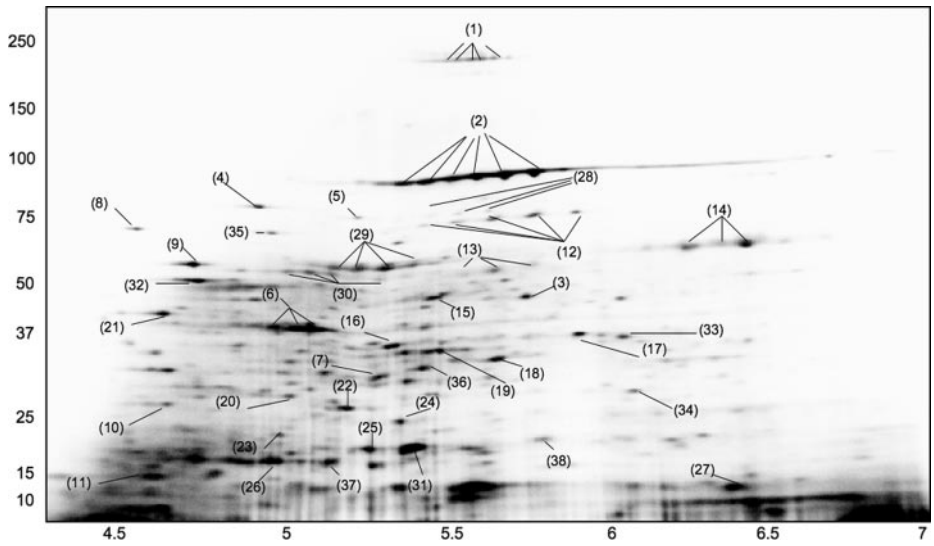

FIG. 4. **Spore proteome of *B. anthracis* 34F2 Sterne.** Protein identifications associated with the spot numbers (*1–38*) are shown in Table II. Representative gels from four experiments are shown.

primary fragment to generate His_6-tagged proteins. The synthesized His_6-tagged proteins from the previous step were purified by nickel-nitrilotriacetic acid Superflow 96 kit (Qiagen) according to the manufacturer's instructions. TAP fragments were subsequently filter-purified to remove free nucleotides and other impurities.

The resulting transcriptionally active products were used as a template for cell-free *in vitro* translation reactions using the Rapid Translational System (RTS) ProteoMaster by Roche Applied Science, and high levels of protein were recovered and then purified. The level of protein obtained was typically 75–100 mg/50-ml reaction. To verify the identity and purity of each of the synthesized proteins, the proteins were resolved by one-dimensional SDS-PAGE and stained with Coomassie Blue dye. The identities of the proteins were confirmed with a QSTAR Hybrid LC/MS/MS system.

ELISA—The basic sandwich ELISA technique was conducted with the proteins generated by *in vitro* TAP and RTS approaches. Each of the proteins (100 μl containing 1 μg/ml diluted protein) were coated onto the 96-well plate and incubated overnight at 4 °C. The plates were washed with 2× PBS with Tween 20, and the unbound antigens (proteins) were discarded by flicking the contents of the plate. Each of the wells was blocked for 90 min at 37 °C by adding 200 μl of 3% skim milk prepared in phosphate-buffered saline with 0.05% Tween 20 (PBST). The blocking buffer was discarded, and the plates were tapped dry on a paper towel. This was followed by adding 100 μl of rabbit serum sample diluted in blocking buffer (for generation of rabbit immune sera, see below) and incubating for 1 h at 37 °C. Then the washing steps were repeated followed by addition of an affinity-purified antibody, peroxidase-labeled goat anti-rabbit IgG heavy + light, and incubating for 1 h at 37 °C. The plates were finally washed with PBST and stained with 3,3′,5,5′-tetramethylbenzidine membrane peroxidase substrate until the chemiluminescent immunoreactive spots appeared in the wells. The 96-well plates were read on an ELISA plate reader at a wavelength of 405 nm.

Generation of Rabbit Immune Sera—Rabbit immune sera were generated against *B. anthracis* 34F2 Sterne full germination-deficient (Δ*gerH*) (44) whole spores and purified exosporium. The spores of Δ*gerH* strain were prepared as mentioned above under "Bacterial Spore Preparation" except that the spores used to immunize the rabbits were not purified by renograffin. Exosporium fragments were recovered by pulse-sonicating *B. anthracis* 34F2 Sterne spores (preparation mentioned above) for 15 s for four times at level 4 with a Model 100 cell dismembrator (Fisher Scientific). The sample was subjected to low speed centrifugation at 25 °C for 5 min. The supernatant containing the exosporium was filtered (0.45 μm) to remove all the remaining spore materials and stored at −20 °C for further use. We confirmed by TEM examination that this procedure removed the majority of the exosporium fragments without detectable damage to the remaining spore (data not shown). Three outbred rabbits were immunized per antigen by intradermal injection. To generate immune sera each rabbit received either 100 μl of 1×10^7 of Δ*gerH* spores or 100 μg of filtered exosporium. Nine boosts per antigen were given to each of the rabbits, *i.e.* a biweekly boost schedule for the first 6 months followed by a monthly boost for the next 3 months. The immune sera obtained from three rabbits in both the groups, against *B. anthracis* 34F2 exospore and Δ*gerH* whole spore, were pooled, respectively, for the Western blot analysis. For controls, corresponding antisera were collected from unimmunized rabbits that were injected with phosphate-buffered saline.

To compare the cross-reactivity of spore proteins of the 10 *Bacillus* strains between immune sera generated against *B. anthracis* 34F2 Sterne Δ*gerH* whole spores, exosporium, and killed ungerminated *B. anthracis* spores, we used rabbit immune sera generated against irradiated (killed) ungerminated *B. anthracis* Ames spores, a generous gift from Dr. Arthur Freidlander (United States Army Medical Research Institute for Infectious Diseases) (45). For the purpose of this study the four sera have been abbreviated as follows: rabbit anti-gerH whole spore sera (RAGSS), rabbit anti-exosporium sera (RAESS), rabbit anti-whole spore (ungerminated) sera (RAWSS), and rabbit anti-naïve sera (RANS).

Western Blot Analysis of 2D Separated Proteins—Proteins on 2D gels of all 10 *Bacillus* strains were transferred to PVDF membranes using Towbin buffer (25 mM Trizma (Tris base), 190 mM glycine, and 20% methanol) at 100 V for 30 min. After the transfer, the PVDF membrane was blocked with 3% skim milk prepared in PBST for 1 h followed by three 5-min washes with PBST. The blots were then probed for 1 h with 1:50,000 dilutions of RAGSS, RAESS, RAWSS, or RANS prepared in 3% skim milk. After probing with the primary antibody, the blots were washed three times with PBST and probed with a 1:25,000 dilution of an affinity-purified antibody, peroxidase-labeled goat anti-rabbit IgG heavy + light, for 1 h. The blots were finally washed with PBST and stained with 3,3′,5,5′-tetramethylbenzidine membrane peroxidase substrate until the chemiluminescent immunoreactive spots appeared on the blot. The blots were dried, and the images were captured using a ChemiDoc XRS system (Bio-Rad). Three replicate blots for each of the antigens for all 10 strains were generated.

TABLE II
Proteins identified from B. anthracis 34F2 Sterne spore
The spot number indicates the corresponding protein spot in the 2D electrophoretic map shown in Fig. 4.

Spot no.	Locus tag (GBAA)	Accession no. (gi)	Protein	No. of peptides matched	Sequence coverage	Theoretical molecular mass (kDa)/pI
					%	
Cell wall/surface						
1	1094	47526368	Wall-associated protein, putative (RhsA)	16	32	249.01/5.97
2	0887	47526173	S-layer protein EA1 precursor (Eag)	21	31	91.36/5.70
3	0252	47525509	Alanine racemase (Dal-1/Alr)	12	36	43.66/5.51
Translation						
4	0107	47525363	Translation elongation factor G (FusA)	25	36	76.33/4.91
5	3950	47529240	Translational initiation factor (InfB)	23	33	75.75/5.03
6	0108	47525364	Translation elongation factor Tu (Tuf)	28	53	42.93/4.93
7	3964	47529254	Translation elongation factor Ts (Tsf)	22	45	32.30/5.25
Protein folding assembly						
8	4539	47529836	Molecular chaperone DnaK	16	40	65.76/4.65
9	0267	47525527	Chaperonin GroEL	23	31	57.43/4.79
10	4540	47529837	Hsp70 cofactor, GrpE protein	11	61	21.52/4.56
11	0266	47525525	Co-chaperonin GroES	7	37	10.06/4.69
Energy metabolism						
12	4843	47530138	Pyruvate kinase (PykA-2)	14	55	62.10/5.20
13	0309	47500720	Δ^1-Pyrroline-5-carboxylate dehydrogenase	22	31	56.22/5.43
14	0008	47525264	Inosine-5-monophosphate dehydrogenase (GuaB)	17	47	52.37/6.30
15	4385	47529680	Dihydrolipoamide dehydrogenase (BfmbC)	33	51	50.77/5.53
16	5367	47530678	Phosphoglycerate kinase (Pgk)	21	61	42.29/5.14
17	5716	47531053	Adenylosuccinate synthetase (PurA)	18	60	47.42/5.57
18	4184	47529480	Pyruvate dehydrogenase, E1α (PdhA)	14	63	41.44/5.52
19	5369	47530679	Glyceraldehyde-3-PO_4 dehydrogenase (Gap-2)	10	35	35.82/5.37
20	4183	47529479	Pyruvate dehydrogenase, E1β (PdhB)	12	32	35.22/4.75
21	5364	47530674	Enolase (Eno)	13	43	46.41/4.66
Amino acid biosynthesis						
22	5387	47778397	Thioredoxin reductase B (TrxB)	9	47	34.66/5.16
23	0131	47525387	Adenylate kinase (Adk)	6	35	23.74/4.91
Sporulation						
24	0803	47526092	Coat protein JC (CotJC)	8	51	21.65/5.15
25	0053	47525307	Stage V sporulation protein T (SpoVT)	21	38	19.69/5.09
26	1238	47526503	Spore coat protein Z (CotZ-2)	13	31	16.84/4.71
27	4898	47530192	Small acid-soluble spore protein B (SspB)	6	53	6.80/6.13
Nucleotide metabolism						
28	3944	47529234	Polyribonucleotide nucleotidyltransferase (Pnp)	11	36	78.20/5.16
29	5583	47530904	CTP synthase (CtrA)	25	34	59.75/5.28
30	5549	47530869	ATP synthase, F_1 (AtpA)	25	39	54.64/5.30
Cell signaling						
31	4499	47529794	Mn-superoxide dismutase (SodA-1)	7	58	22.66/5.31
Cellular process						
32	0344	47525611	Alkyl hydroperoxide reductase, F (AhpF)	24	35	54.81/4.93
33	2541	47502979	ABC transporter, ATP-binding protein	8	40	25.00/5.93
34	5222	47505676	ABC transporter, ATP-binding protein	15	42	37.26/6.18
Hypothetical protein						
35	1267	47526534	Hypothetical protein	23	31	73.25/5.29
36	0029	47525285	Hypothetical protein	11	39	31.76/5.38
37	0020	47525275	Hypothetical protein	9	59	11.86/5.17
38	0145	47525401	Hypothetical protein	10	45	17.45/5.82

TABLE III
Matching summary of protein spots expressed in two-dimensional electrophoretic maps of spores of B. anthracis and closely related strains
Spots matched to every gel, 72%.

Strains	Clades	Spot count	Spots matched	Match rate
				%
B. anthracis Sterne 34F2	1	370	370	100
B. thuringiensis BGSC 4AJ1	1	390	322	83
B. cereus 1293	1	337	269	80
B. cereus BGSC 6E1	1	255	212	83
B. cereus ATCC 10987	1	231	190	82
B. cereus ATCC 10876	2	236	184	78
B. thuringiensis BGSC 4Y1	2	294	229	78
B. cereus m1550	3	228	185	81
B. mycoides DSMZ 2048	3	378	298	79
B. pseudomycoides DSMZ 12442	Outlier	246	184	75

RESULTS

Bioinformatics-based Screening of Conserved B. anthracis Spore Proteins—To identify conserved proteins we aligned the 5,567 predicted proteins in the annotated genome sequence of *B. anthracis* Ames ancestor (RefSeq accession numbers NC_007530, NC_007323, and NC_007322) against a database of the predicted proteomes of the published *B. cereus s.l.* genomes available at the start of the study in 2004 (*B. cereus* G9241 (6), *B. cereus* ATCC 10987 (4), *B. cereus* E33L (5), *B. cereus* 14579 (3), and *B. thuringiensis* serovar *konkukian* 97-27 (3)) using the BLASTP tool (46, 47). Using a cutoff E-value of 10^{-5} for the significance of the predicted match, 2,800 proteins were found to be conserved in all six *B. cereus* group genomes (Fig. 2). We then cross-referenced this set against more than 750 endospore and exosporium constituent proteins of *B. anthracis* identified by multidimensional chromatography and tandem mass spectroscopy (29) to obtain a subset of 560 proteins. The proteins in this list were then ranked manually as likely antigenic candidates based on (i) annotation and literature references that indicated known spore structural components or late stage sporulation proteins, (ii) bioinformatics-based prediction of subcellular localization using PSORTb (48) that indicated a membrane-associated or exported protein, and (iii) a peptide length greater than 100 amino acids. The ORFs encoding the top 200 candidate proteins were selected for *in vitro* gene-specific expression (Fig. 2).

The 200 target ORFs were then amplified and expressed using the high throughput cell-free TAP fragment system. The resulting transcriptionally active products were used as a template for cell-free *in vitro* translation reactions using the RTS ProteoMaster by Roche Applied Science, and high levels of protein were recovered and then purified. Of the 200 ORFs, 192 proteins were synthesized successfully, and the identities were verified by mass spectroscopy.

These 192 synthesized proteins were subjected to ELISA against RAGSS, RAESS, and RANS. 117 of the synthesized proteins did not react with any one of the immune sera (Table I). Of the 75 proteins that did react, 23 proteins reacted with both RAGSS and RAESS. None of these 23 proteins had been previously identified as *B. anthracis* spore surface proteins but were instead mainly ABC transporters, proteins of unknown function (annotated as "hypothetical," *e.g.* GBAA2334, GBAA1237, and GBAA1334), sporulation regulators (SpoIIIAG and SpoVAD), and housekeeping proteins. 37 proteins reacted with sera generated from RAGSS, suggesting that these proteins are present either in the spore coat, cortex, and/or core (Table I). Only 15 proteins reacted with RAESS. RAESS-reactive proteins included small acid-soluble spore protein B (SspB), coat protein JB (CotJB), and alanine racemases (Dal-1, also known as Alr), which have previously been identified as spore constituents (Table I) (2, 14, 23, 32). There were three proteins of undetermined function (GBAA5641, GBAA2292, and GBAA1172) that reacted only with RAESS (Table I).

Microvariation in B. cereus s.l. Spore Morphology—In a parallel screening effort, spores from 10 *B. cereus* group strains were produced by growth under similar conditions (Supplement 2) for comparative proteomics analysis. The spore preparations were examined by TEM as a quality control step (see "Experimental Procedures"; Fig. 3, *panel I*) presenting an opportunity for comparative analysis of spore ultrastructure. On average, 127 spores per *Bacillus* strain were examined. Only longitudinally sectioned spores were measured to obtain the core and interspace dimensions. A representative figure of the calculations is shown in Supplement 3. All *Bacillus* spores examined display an elongated olive shape with an extension at each pole. Most of the spores in clade 1 were structurally similar with their core measurements ranging from 1.003 to 1.207 μm in length and from 0.511 to 0.658 μm in width (Fig. 3, *panel II*). The cores of the *Bacillus* spores of clades 2 and 3 were on average smaller than spores in clade 1, although the variance of the measurements was too high to draw statistically significant conclusions. We observed other structural variations. In spores of *B. thuringiensis* BGSC 4Y1 (Fig. 3, *panel I*, *G*) the exosporium and the coat have a peculiar ribbon-like structure, which was not observed in any of the other strains. Furthermore on higher magnification, as expected, *B. thuringiensis* BGSC 4Y1 spores showed the presence of crystalline structures in the interspace region. However, we did not see crystals in the spores of *B. thuringiensis* BGSC 4AJ1. In *B. pseudomycoides* DSMZ 12442 (Fig. 3, *panel I*, *J*) the interspace (IS) region is almost absent as the exosporium wraps tightly around the spore coat even at the pole region. The average IS length in *B. pseudomycoides* DSMZ 12442 is 0.045 μm with a maximum of 0.192 μm at the elongated portion of the spores. More images of spores of *B. thuringiensis* BGSC 4Y1 and *B. pseudomycoides* DSMZ 12442 are shown in Supplement 4. The average IS length in

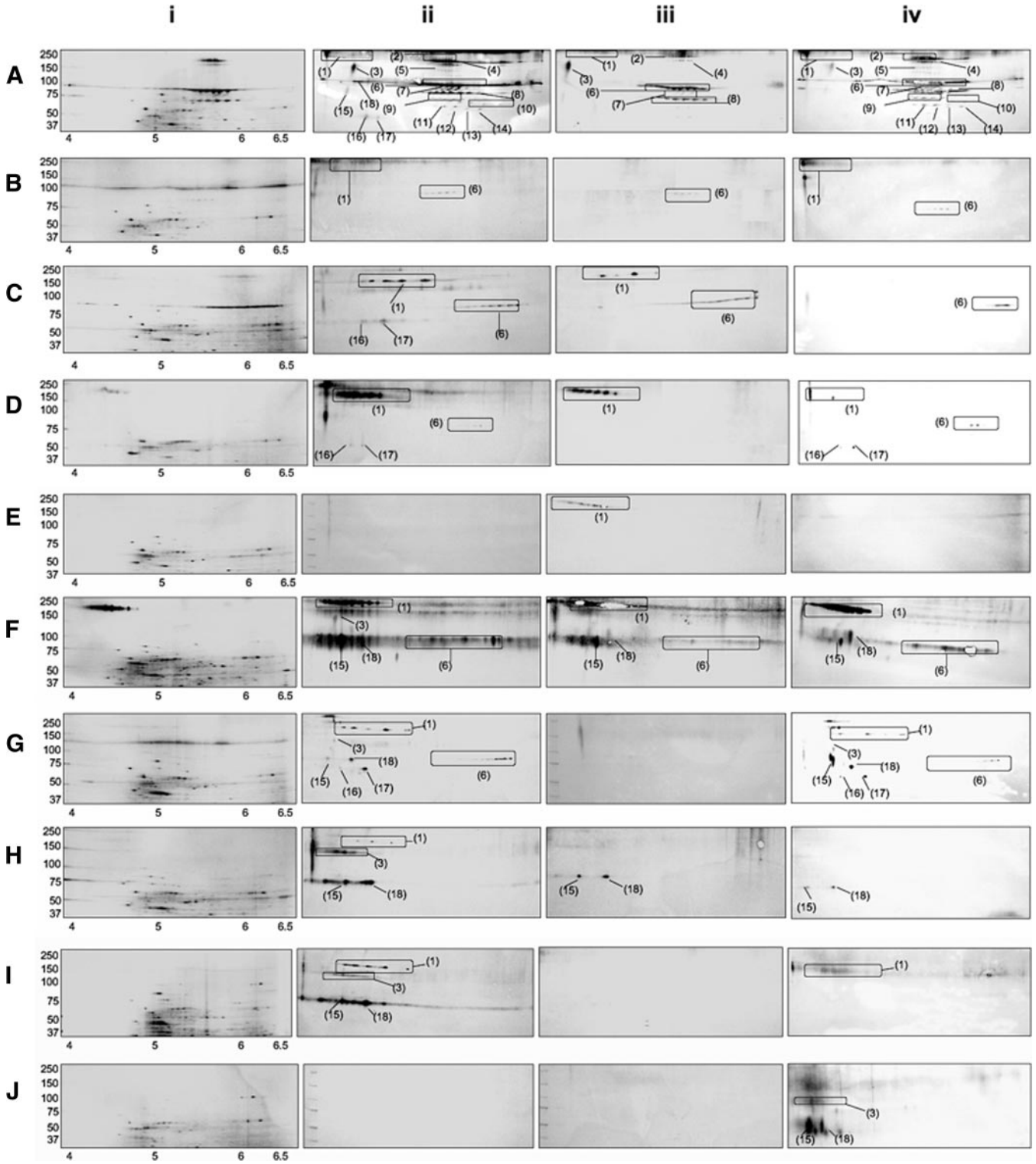

FIG. 5. **Western blot analysis of spore proteins of *B. anthracis* and closely related strains against rabbit immune sera generated against *B. anthracis*.** Only portions of the gels that showed positive reaction to immune sera are shown. *Panel i*, silver-stained gel; *panel ii*, Western blot of spore proteins against rabbit sera to *B. anthracis* Δ*gerH* whole spore (RAGSS); *panel iii*, Western blot of spore proteins against rabbit sera to *B. anthracis* Sterne 34F2 exosporium (RAESS); *panel iv*, Western blot of spore proteins against rabbit sera to *B. anthracis* Ames irradiated ungerminated whole spore (RAWSS). *A*, *B. anthracis* Sterne 34F2; *B*, *B. thuringiensis* BGSC 4AJ1; *C*, *B. cereus* 1293; *D*, *B. cereus* BGSC 6E1; *E*, *B. cereus* ATCC 10987; *F*, *B. cereus* ATCC 10876; *G*, *B. thuringiensis* BGSC 4Y1; *H*, *B. cereus* m1550; *I*, *B. mycoides* DSMZ 2048; *J*, *B. pseudomycoides* DSMZ 12442. Protein identifications associated with the spot numbers (*1–18*) are shown in Table IV.

TABLE IV

Spore proteins identified in B. anthracis and closely related strains from Western blot analysis against rabbit immune sera

The spot number indicates the corresponding protein spot in the 2D electrophoretic Western blots shown in Fig. 5.

Spot no.	Locus tag	Accession no.	Protein	Theoretical molecular mass (kDa)/pI
1	GBAA3601	gi47528887	Conserved repeat domain protein (Crd)	245.09/4.17
2	GBAA1094	gi47526368	Wall-associated protein, putative (RhsA)	249.01/5.97
3	GBAA3677	gi47528961	Aconitate hydratase 1 (AcnA)	99.01/4.8
4	GBAA0052	gi47525306	Transcription-repair coupling factor (Mfd)	134.15/5.36
5	GBAA4157	gi47529455	Pyruvate decarboxylase (Pyc)	128.57/6.0
6	GBAA0887	gi47526173	S-layer protein EA1 precursor (Eag)	91.36/5.70
7	GBAA3944	gi47529234	Polyribonucleotide nucleotidyltransferase (Pnp)	78.20/5.16
8	GBAA4843	gi47530138	Pyruvate kinase (PykA-2)	62.10/5.20
9	GBAA5583	gi47530904	CTP synthase (CtrA)	59.75/5.28
10	GBAA0309	gi47500720	Δ^1-Pyrroline-5-carboxylate dehydrogenase	56.22/5.43
11	GBAA3609	gi47528895	Aldehyde dehydrogenase (DhaS)	53.74/5.4
12	GBAA4184	gi47529480	Pyruvate dehydrogenase, E1α (PdhA)	41.44/5.52
13	GBAA0252	gi47525509	Alanine racemase (Dal-1/Alr)	43.66/5.51
14	GBAA5222	gi47505676	ABC transporter, ATP-binding protein (MetN)	37.26/6.18
15	GBAA4539	gi47529836	Molecular chaperone DnaK	65.76/4.65
16	GBAA0267	gi47525527	Chaperonin GroEL	57.43/4.79
17	GBAA0108	gi47525364	Translation elongation factor Tu (Tuf)	42.93/4.93
18	GBAA0107	gi47525363	Translation elongation factor G (FusA)	76.33/4.91

other *Bacillus* spores ranges from 0.095 to 0.166 μm at the width with the maximum IS ranging from 0.325 to 0.649 μm at the elongated portion of the spores. *B. cereus* m1293, *B. cereus* ATCC 10987, and *B. mycoides* DSMZ 2048 showed larger IS regions compared with other *Bacillus* spores.

Comparative Proteomics Analysis of B. cereus s.l. Spores—We determined the spore proteome of all 10 *Bacillus* strains by generating two-dimensional electrophoresis maps of the proteins extracted from renografffin-purified whole spores grown in modified G medium at 37 °C for about 72 h under shaker condition (Fig. 4). At least four spore preparations of each strain followed by 2D electrophoresis were conducted to confirm the results. A total of 38 proteins from 10 clusters of orthologous groups (cell wall/surface, translation, protein folding and assembly, energy metabolism, amino acid biosynthesis, sporulation, nucleotide metabolism, cell signaling, cellular process, and hypothetical proteins) were identified by mass spectroscopy from spots excised from the *B. anthracis* 34F2 Sterne 2D gel (Table II and Fig. 4).

Comparative protein composition analysis of the spores of all 10 *Bacillus* strains was performed using PDQuest software by generating a match set of all the gels. After normalizing the spots in the gels, each spot in the master gel (*B. anthracis* Sterne 34F2) was matched with the same spot in each of the gels generating a summary of all the spots in all the gels (Table III). More than 300 spots were resolved in *B. anthracis* Sterne 34F2, *B. thuringiensis* BGSC 4AJ1, *B. cereus* m1293, and *B. mycoides* DSMZ 2048, and more than 200 spots were resolved in the 2D gels of the six remaining *Bacillus* strains (Table III). Protein spots of strains in clade 1 had a match rate of $\geq$80% with the protein spots of *B. anthracis* Sterne 34F2 (reference strain). Protein spots of strains in clades 2 and 3 matched $\geq$78%, and the outlier *B. pseudomycoides* DSMZ 12442 matched up to 75% with the reference strain. About 72% of *B. anthracis* Sterne 34F2 protein spots matched with every strain.

Western Blot Analysis against Rabbit Immune Sera—Western blots derived from 10 different spore preparations were probed with RAGSS, RAESS, RAWSS, and RANS (see "Experimental Procedures"). Comparison of the silver-stained 2D electrophoretic maps of each of the 10 *Bacillus* strains was referenced with corresponding Western immunoblots, allowing us to identify sera-reactive spore proteins (Fig. 5). Only the portions of the 2D gels that showed the appearance of sera-reactive protein spots are shown in Fig. 5. Although the limited dynamic range of the proteomics approach imposes severe restrictions on the repertoire of proteins detected, 18 sera-reactive proteins in total were detected from the *Bacillus* strains (Table IV). The spot number indicates the corresponding protein spot in the 2D electrophoretic Western blots shown in Fig. 5.

All 18 protein spots were detected in Western immunoblot of RAGSS (Fig. 5*A*, *panel ii*), and 14 of the spots were detected against RAWSS (Fig. 5*A*, *panel iv*). The only difference between the immunoblots against RAGSS and RAWSS was that the four housekeeping proteins, molecular chaperone (DnaK), chaperone GroEL, translation elongation factor Tu (Tuf), and translation elongation factor G (FusA), were not detected in the immunoblot against RAWSS (Fig. 5*A*, *panels ii* and *iv*). Western blots from six of the 10 different spore preparations, *i.e. B. anthracis* Sterne 34F2, *B. thuringiensis* strains BGSC 4AJ1 and BGSC 4Y1, and *B. cereus* strains BGSC 6E1, ATCC 10987, and ATCC 10876, showed very similar results when probed with RAGSS and RAWSS (Fig. 5, *A*, *B*, and *D–G*, *panels ii* and *iv*), although the clade 1 strain *B.*

TABLE V

Sera-reactive conserved Bacillus spore proteins

+, spots present in Western immunoblot against anti-*B. anthracis* 34F2 Δ*gerH* spore; ●, spots present in Western immunoblot against anti-*B. anthracis* 34F2 exospore; ▲, spots present in Western immunoblot against anti-whole spore (irradiated ungerminated *B. anthracis* Ames). Ba, *B. anthracis*; Bt, *B. thuringiensis*; Bc, *B. cereus*; Bm, *B. mycoides*; Bp, *B. pseudomycoides*.

Spot no.[a]	Locus (GBAA)	Protein	Theoretical molecular mass (kDa)/pI	Ba 34F2	Bt 4AJ1	Bc 1293	Bc 6E1	Bc 10987	Bc 10876	Bt 4Y1	Bc 1550	Bm 2048	Bp 12442
1	3601	Conserved repeat domain protein (Crd)	245.09/4.17	+●▲	+▲	+●	+●▲	●	+●▲	+▲	+	+▲	
2	1094	Wall-associated protein, putative (RhsA)	249.01/5.97	+●▲									
3	3677	Aconitate hydratase 1 (AcnA)	99.01/4.8	+●▲					+	+▲	+	+	▲
4	0052	Transcription-repair coupling factor (Mfd)	134.15/5.36	+●▲									
5	4157	Pyruvate decarboxylase (Pyc)	128.57/6.0	+●▲									
6	0887	S-layer protein EA1 precursor (Eag)	91.36/5.70	+●▲	+●▲	+●▲	+▲		+●▲	+▲			
7	3944	Polyribonucleotide nucleotidyltransferase (Pnp)	78.20/5.16	+●▲									
8	4843	Pyruvate kinase (PykA-2)	62.10/5.20	+●▲									
9	5583	CTP synthase (CtrA)	59.75/5.28	+▲									
10	0309	Δ^1-Pyrroline-5-carboxylate dehydrogenase	56.22/5.43	+▲									
11	3609	Aldehyde dehydrogenase (DhaS)	53.74/5.4	+▲									
12	4184	Pyruvate dehydrogenase, E1α (PdhA)	41.44/5.52	+▲									
13	0252	Alanine racemase (Dal-1/Alr)	43.66/5.51	+▲									
14	5222	ABC transporter, ATP-binding protein (MetN)	37.26/6.18	+▲									
15	4539	Molecular chaperone DnaK	65.76/4.65	+		+	+▲		+●▲	+▲	+●▲	+	▲
16	0267	Chaperonin GroEL	57.43/4.79	+						+▲			
17	0108	Translation elongation factor Tu (Tuf)	42.93/4.93	+									
18	0107	Translation elongation factor G (FusA)	76.33/4.91	+		+	+▲		+●▲		+●▲	+	▲

[a] The spot number indicates the corresponding protein spot in the 2D electrophoretic map in Fig. 5.

cereus ATCC 10987 did not show any reaction against either sera (Fig. 5*E*, *panels ii* and *iv*).

For the remaining strains, we obtained somewhat inconsistent results probing against the two anti-spore sera. In the Western blots of *B. cereus* ATCC 10876, aconitate hydratase 1 (AcnA) was not detected against RAWSS but was detected against RAGSS (Fig. 5*F*, *panels ii* and *iv*). *B. pseudomycoides* DSMZ 12442 spore proteins did not cross-react with RAGSS, but three proteins, AcnA, DnaK, and FusA, were detected in the immunoblot against RAWSS (Fig. 5*J*, *panels ii* and *iv*). In the remaining three spore preparations (*B. cereus* m1293 and m1550 and *B. mycoides* DSMZ 2048) the proteins detected in the immunoblots against RAGSS and RAWSS showed different patterns (Fig. 5, *C*, *H*, and *I*, *panels ii* and *iv*). Neither of the three strains, *B. thuringiensis* BGSC 4Y1, *B. mycoides* DSMZ 2048, and *B. pseudomycoides* DSMZ 12442, reacted with the sera generated against the exosporium fraction of *B. anthracis* Sterne 34F2 (Fig. 5, *G*, *I*, and *J*, *panel iii*). In the other seven strains, one to seven proteins reacted against RAESS (Fig. 5, *A–F* and *H*, *panel iii*).

Comparison of all the proteins in the Western immunoblots obtained from all 10 *Bacillus* strains revealed six proteins that were sera-reactive in most of the *Bacillus* strains: a conserved repeat domain protein family, given the name in this work as "Crd," S-layer protein EA1 precursor (Eag), DnaK, GroEL, Tuf, and FusA (Table V). Two of the proteins identified by the proteomics screen, Dal-1 and an ABC transporter, ATP-binding protein (MetN), overlapped the list of potential targets from the bioinformatics-based screening (Tables I and V).

Amino Acid Sequence Conservation of Cross-reacting Spore Antigens—We performed a translated BLAST search against the genome sequences of the *B. cereus* strains to determine whether failure to cross-react to the antisera derived from *B. anthracis* spore challenge in other *B. cereus* group strains was caused by gene loss or sequence divergence (Table VI and Supplement 5). In some cases strains lack an ortholog of the *B. anthracis* protein. For instance, cell wall-associated protein (RhsA) only has an ortholog with sequence similarity greater than 75% in *B. cereus* ATCC 10987, BGSC 6E1, and m1550 although RhsA from either of these

TABLE VI

Amino acid sequence similarity of orthologs in B. cereus s.l.

Cutoff, at least 50% protein identity over 100 amino acids. Protein identity is represented in percent. The length of the amino acid sequence hit is presented in parentheses. The bold portions represent the correlation of sera-reactive conserved *Bacillus* spore proteins in Table V. aa, amino acids; Ba, *B. anthracis*; Bt, *B. thuringiensis*; Bc, *B. cereus*; Bm, *B. mycoides*; Bp, *B. pseudomycoides*.

Accession no.	Locus tag	Protein	Bt4AJ1	Bc1293	Bc6E1	Bc10987	Bc10876	Bt4Y1	Bc1550	Bm2048	Bp12442
gi47528887	GBAA3601	Conserved repeat domain protein (Crd), 2357 aa	**72.36 (1616)**	**71.87 (1616)**	**71.19 (1616)**	**69.71 (1616)**	**71.19 (1616)**	**90.65 (213)**	**69.62 (1616)**	**69.34 (1616)**	—
			80.33 (2326)	**79.16 (2326)**	**79.50 (2326)**	**77.09 (2325)**	**78.39 (2326)**	**93.82 (468)**	**77.80 (2326)**	**77.80 (2326)**	
								91.39 (1729)			
gi47526368	GBAA1094	Wall-associated protein, putative (RhsA), 2223 aa	—	—	76.81 (265)	55.42 (162)	—	—	57.14 (209)	—	—
						50.19 (237)			85.45 (430)		
						76.47 (318)					
						82.66 (2210)					
						86.82 (2107)					
gi47528961	GBAA3677	Aconitate hydratase 1 (AcnA), 906 aa	100.00 (906)	99.67 (906)	100.00 (906)	99.56 (906)	**99.89 (906)**	**99.67 (906)**	**99.67 (906)**	**97.68 (906)**	**97.02 (906)**
gi47525306	GBAA0052	Transcription-repair coupling factor (Mfd), 1175 aa	99.91 (1175)	98.95 (475)	99.83 (578)	99.74 (1175)	97.96 (1175)	95.64 (320)	97.96 (1175)	97.62 (1175)	89.50 (475)
				98.04 (712)	99.50 (602)			97.10 (826)			97.53 (687)
gi47529455	GBAA4157	Pyruvate decarboxylase (Pyc), 1147 aa	94.57 (220)	98.98 (489)	99.30 (1147)	99.13 (1147)	97.53 (1092)	99.13 (1147)	98.10 (209)	97.96 (489)	55.38 (128)
			99.57 (939)	98.80 (663)					98.29 (937)	96.84 (663)	94.39 (1087)
gi47526173	GBAA0887	S-layer protein EA1 precursor (Eag), 861 aa	**59.88 (171)**	**53.26 (182)**	**52.11 (210)**	58.72 (171)	**52.94 (135)**	**59.88 (171)**	51.53 (195)	60.47 (171)	—
			51.53 (195)	**59.30 (171)**	**55.78 (198)**	53.16 (188)	**53.10 (144)**	**52.11 (210)**		54.27 (198)	
			56.35 (195)	**60.13 (152)**	**88.53 (861)**	56.12 (195)		**53.77 (206)**		57.14 (195)	
			55.78 (198)	**56.12 (195)**		56.28 (198)		**55.78 (198)**			
			99.88 (861)	**87.69 (267)**				**88.79 (854)**			
				83.73 (165)							
				87.24 (383)							
gi47529234	GBAA3944	Polyribonucleotide nucleotidyltransferase (Pnp), 711 aa	99.71 (693)	99.28 (693)	100.00 (115)	99.14 (693)	99.42 (693)	99.86 (693)	99.42 (693)	96.69 (693)	95.39 (693)
					99.83 (578)						
gi47530138	GBAA4843	Pyruvate kinase (PykA-2), 584 aa	100.00 (584)	99.63 (540)	100.00 (584)	99.66 (584)	99.66 (584)	99.55 (223)	96.05 (584)	94.76 (190)	98.46 (584)
								98.85 (346)		97.77 (403)	
gi47530904	GBAA5583	CTP synthase (CtrA), 534 aa	99.63 (534)	99.44 (534)	99.63 (534)	99.25 (534)	98.69 (534)	99.02 (509)	98.88 (534)	98.50 (534)	97.76 (534)
gi47500720	GBAA0309	Δ^1-Pyrroline-5-carboxylate dehydrogenase, 514 aa	100.00 (514)	100.00 (514)	100.00 (514)	99.81 (514)	99.42 (514)	99.81 (514)	99.49 (197)	98.72 (233)	96.31 (514)
									99.37 (317)	95.73 (280)	
gi47528895	GBAA3609	Aldehyde dehydrogenase (DhaS), 493 aa	64.96 (487)	65.16 (487)	64.96 (487)	65.16 (487)	63.89 (492)	65.16 (487)	64.56 (157)	64.55 (487)	65.37 (487)
			100.00 (493)	98.58 (493)	100.00 (493)	98.58 (493)	98.58 (493)	98.99 (493)	65.73 (320)	97.57 (493)	91.76 (169)
									98.58 (493)		95.08 (324)
gi47529480	GBAA4184	Pyruvate dehydrogenase, E1α (PdhA), 372 aa	100.00 (370)	100.00 (370)	100.00 (370)	100.00 (370)	98.92 (370)	100.00 (367)	98.92 (370)	99.69 (323)	99.73 (370)
gi47525509	GBAA0252	Alanine racemase (Dal-1/Alr), 388 aa	99.74 (388)	99.23 (388)	99.74 (388)	99.49 (388)	96.40 (388)	99.23 (388)	96.66 (388)	95.89 (388)	87.40 (388)
gi47505676	GBAA5222	ABC transporter, ATP-binding protein, methionine transport (MetN) 340 aa	100.00 (340)	99.71 (340)	94.41 (340)	99.41 (340)	99.12 (340)	100.00 (340)	99.12 (340)	98.43 (254)	88.56 (340)

TABLE VI—*continued*

Accession no.	Locus tag	Protein	Bt4AJ1	Bc1293	Bc6E1	Bc10987	Bc10876	Bt4Y1	Bc1550	Bm2048	Bp12442
gi47529836	GBAA4539	Molecular chaperone DnaK, 610 aa	100.00 (610)	**98.20 (610)**	**98.79 (164)** **99.56 (451)**	98.04 (610)	**99.67 (610)**	**99.84 (610)**	**100.00 (231)** **99.73 (368)**	**98.69 (610)**	**95.91 (610)**
gi47525527	GBAA0267	Chaperonin GroEL, 543 aa	99.81 (530)	99.81 (530)	99.81 (530)	97.24 (180) 100.00 (353)	99.25 (530)	**99.25 (530)**	99.25 (530)	97.93 (530)	98.31 (530)
gi47525364	GBAA0108	Translation elongation factor Tu (Tuf), 394 aa	100.00 (394)	99.75 (394)	100.00 (394)	99.75 (394)	100.00 (394)	100.00 (394)	100.00 (146) 100.00 (247)	97.39 (305)	98.73 (393)
gi47525363	GBAA0107	Translation elongation factor G (FusA), 691 aa	100.00 (691)	**99.42 (691)**	**100.00 (689)**	99.42 (691)	**99.42 (691)**	99.42 (691)	**99.42 (691)**	**98.12 (691)**	**98.70 (691)**

three strains showed no cross-reaction with any of the sera (Fig. 5, *A*, *D*, *E*, and *H*). The diverged *B. pseudomycoides* DSMZ 12442 genome lacks apparent orthologs to a number of *B. anthracis* spore proteins (Table VI). On the other hand, some proteins that were 100% identical in amino acid sequence to *B. anthracis* proteins did not exhibit detectable cross-reaction, for example *B. thuringiensis* BGSC 4AJ1 AcnA, PykA-2, Δ^1-pyrroline-5-carboxylate dehydrogenase, DhaS, PdhA, MetN, DnaK, Tuf, and FusA (Table VI and Supplement 5).

DISCUSSION

In this project we used two complementary approaches to select spore antigens that could produce antibodies cross-reactive against a broad range of *B. cereus* group spores. For the first method we used *in silico* filters to produce a list of 200 proteins by their conservation across five genomes. In the second approach we used a comparative proteomics analysis to detect cross-reacting spore antigens (Fig. 6). Both strategies have their drawbacks. Sequence-based analyses alone cannot predict the composition of the *Bacillus* spore especially considering the likely variability introduced by transcriptional and translational regulation of protein expression. However, any proteomics approach has inherent bias toward identification of abundant proteins. Combining both the approaches produced a relatively small list of potential conserved candidate antigens.

The sera we used for challenging the proteins were taken from rabbits injected with *B. anthracis* exosporium (RAESS) and *B. anthracis* whole spore germination mutants (RAGSS) (44). Because *B. anthracis* has multiple genetic loci dedicated to triggering germination (49, 50) there is a possibility that a small portion of the spores may germinate *in vivo* and elicit responses to vegetative cell antigens. However, we found that results using RAGSS were mostly similar to those using sera from killed whole spores (RAWSS), indicating that germination *in vivo* is not a major issue for this Δ*gerH* mutant.

We identified 75 spore *B. anthracis* proteins from the bioinformatics-based screen that reacted against RAESS or RAGSS and 18 from the proteomics screen that reacted against RAESS, RAGSS, or RAWSS (Fig. 6). Only two protein candidates were found to be common to both approaches (Dal-1 and MetN). The activity of Dal-1 is higher in spores than in the vegetative cells (49, 51, 52), and the protein has been found to be a germination inhibitor in the exosporium (29, 51). MetN (methionine import ATP-binding protein) is responsible for energy coupling to the transmembrane transport system. MetN has not been reported as an immunogenic protein in any previous *Bacillus* studies, although immunogenic ABC transporters are being considered as potential vaccine candidates against several pathogens such as *Helicobacter*, *Burkholderia*, *Streptococcus*, *Chlamydia*, and *Rickettsia* species (53–58). *B. anthracis* spore antisera did not cross-react against the Dal-1 and MetN orthologs from the proteomes of

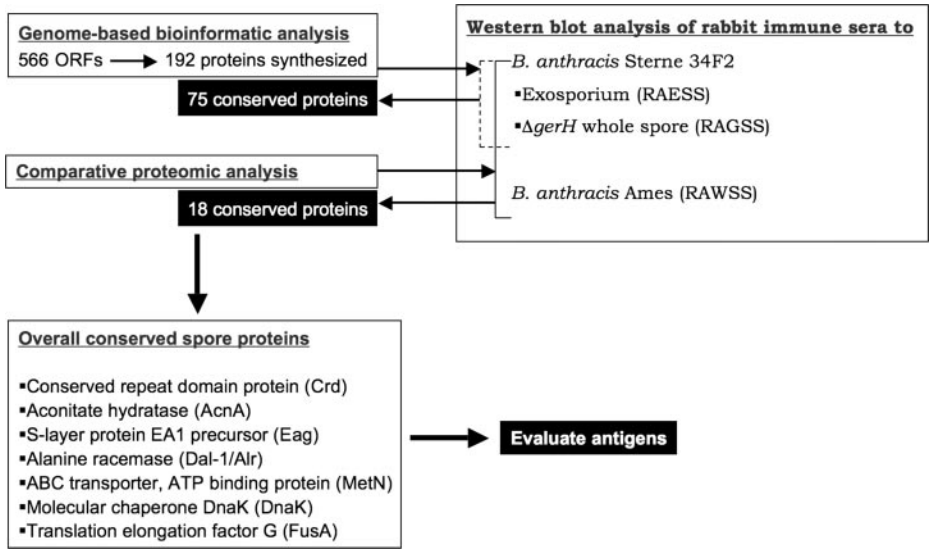

FIG. 6. **Schematic representation of the results obtained through the study design to obtain potential vaccine candidates.**

the nine other strains tested. Our interpretation of these results is that the spore surface components proteins are in low abundance and/or elicit immune responses that in general cannot be detected by the relatively insensitive 2D gel electrophoresis. This is backed up by other studies (37, 59) that failed to detect several known spore proteins on the 2D protein gels. In some cases, the low abundance of the protein is the likely reason for the failure to detect in our studies, for instance, the Exs exosporium components (20, 36, 60, 61) and the recently discovered SoaA protein (62). In the case of the major exosporium component BclA (36, 63), a lack of trypsin cleavage sites precludes identification.

Four of the 18 cross-reacting proteins from the proteome-based screen (AcnA, Eag, DnaK, and FusA), were identified in *B. anthracis* spore lysates by Liu *et al.* (29). The remaining 18 proteins are not exclusively exosporium or endospore structural components but rather important core metabolic proteins presumably included to facilitate rapid growth upon germination. Translation elongation factors (Tuf and FusA) and chaperones (DnaK and GroEL) are examples of this class of spore proteins. Another class of the proteins listed in Table IV are those that are likely debris from the lysed mother cells adhered onto the surface of the spores. The Eag protein has been shown to be expressed only at the stationary phase of the vegetative cell (64). The RhsA protein probably also falls under the category of an incidental spore component. Another cross-reactive protein identified from this screen is the Crd protein. This large protein with 2,358 amino acids (molecular mass/pI, 244.9 kDa/4.17) contains 15 imperfect repeats of a 132-residue domain (PFAM (Protein Family database) accession number PF01345) that is found in three other *B. anthracis* proteins, GBAA1618 (molecular mass/pI, 523.3 kDa/4.06), GBAA3725 (molecular mass/pI, 219.8 kDa/4.2), and GBAA3721 (molecular mass/pI, 7.8 kDa/5.71). However, only Crd was detected in the protein gels. Genes encoding proteins with this motif are common in the *B. cereus* group but not found in the outgroup endospore-forming bacteria *B. pseudomycoides* DSMZ 12442 and *B. subtilis.* Proteins with multiple PF01345 domains appear in bacteria as diverse as *Chlamydia trachomatis* and the archaebacterium *Methanobacterium.* Crd lacks a classic type II signal sequence and LPXTG binding motif suggesting that it is neither cell wall- nor membrane-associated. The broad range of spores of *B. cereus* that cross-react to Crd suggests a promising candidate, and further investigation on the localization of this protein is necessary.

Several other researchers have demonstrated the power of proteomics analysis in a high throughput mode, including combining genome-based bioinformatics approaches (29, 37, 59). Of the proteins discussed in this report, Dal-1, Eag, Tuf, and FusA have been previously identified as immunogenic proteins from members of the *B. cereus s.l.* group (37, 59). The remaining four immunogenic proteins, MetN, Crd, AcnA, and DnaK, have not been reported previously.

One confounding experimental variable in the proteomics studies was the unexpected lack of cross-reaction to endospore antisera raised against *B. anthracis* by *B. cereus* ATCC 10987. *B. cereus* ATCC 10987 is much more closely related to *B. anthracis* than clade 2 and clade 3 strains that did cross-react to the sera. From our genomics analysis of the data it is clear that highly conserved orthologs of almost all the targets (in some proteins >97% similarity) found by the study are present in the genome of *B. cereus* ATCC 10987. Other strains that encode proteins with sequence very similar or identical to proteins in *B. anthracis* that elicit an immune response also do not cross-react to the sera. Expression patterns of the potential cross-reacting proteins during sporulation may vary in different genetic backgrounds, resulting in low abundance of the proteins in the electrophoretic maps. The design of this study, where the proteomes of 10 phylogenetically diverse strains were profiled, to a certain extent

mitigates against unusual results in one or two individual strains.

Despite a high degree of chromosomal gene conservation and synteny among *B. cereus s.l.* genomes (2, 7), there are significant strain-specific differences in spore coat and endospore proteins, which may contribute to the survival and adaptation in host invasion (65). It is unlikely that the mammalian adaptive immune system is the main agent for selection as most members of this group would appear to infect insects and other invertebrates primarily (66).

Comparative analysis of the spore proteomes of the 10 diverse strains of the *B. cereus* group has shown that it is possible to distinguish between all these strains based on their 2D profiles with spots unique to each strain and only 72% overall matched spots. Ultrastructural analysis of the spores also showed variations, particularly in the exosporium, core dimension, and interspace regions. With further cross-reaction studies against other *Bacillus* species it may be possible to select a mixture of antigens (or a multiepitope antigen) specific for *B. cereus* group spores. Fluorescent tagged antibodies raised against these proteins could have a role as an environmental or clinical detection tool for *B. cereus s.l.* strains.

Acknowledgments—We sincerely thank Lieutenant M. Weiner for providing the *gerH* mutant *B. anthracis* 34F2 Sterne strain; Drs. D. Roth, J. He, and A. Greener for performing the TAP analysis; Drs. N. Nguyen and W. Hicks for mass spectrometry analysis; L. Liang for facilitating the generation of rabbit immune sera; and Dr. L. Baillie and S. Hibbs for providing the *B. anthracis* 34F2 Sterne spore suspension to inject rabbits. We appreciate the generosity of Dr. A. M. Freidlander for providing the RAWSS.

* This work was supported by Joint Science and Technology Office-Chemical and Biological Defense/Defense Threat Reduction Agency Grant 9Y0010_05_NM_B (to T. D. R.).

S The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

The whole genome shotgun data reported in this paper have been submitted to GenBank™ with NCBI Genome Project IDs 29709, 29713, 29671, 29651, 29657, 29649, 29701, and 29707.

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