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Identification of proteins in the exosporium of *Bacillus anthracis*

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Spores of *Bacillus anthracis*, the causative agent of anthrax, possess an exosporium. As the outer surface layer of these mature spores, the exosporium represents the primary contact surface between the spore and environment/host and is a site of spore antigens. The exosporium was isolated from the endospores of the *B. anthracis* wild-type Ames strain, from a derivative of the Ames strain cured of plasmid pXO2$^-$, and from a previously isolated pXO1$^-$, pXO2$^-$ doubly cured strain, *B. anthracis* UM23Cl2. The protein profiles of SDS-PAGE-separated exosporium extracts were similar for all three. This suggests that avirulent variants lacking either or both plasmids are realistic models for studying the exosporium from spores of *B. anthracis*. A number of loosely adsorbed proteins were identified from amino acid sequences determined by either nanospray-MS/MS or N-terminal sequencing. Salt and detergent washing of the exosporium fragments removed these and revealed proteins that are likely to represent structural/integral exosporium proteins. Seven proteins were identified in washed exosporium: alanine racemase, inosine hydrolase, ExsF, CotY, ExsY, CotB and a novel protein, named ExsK. CotY, ExsY and CotB are homologues of *Bacillus subtilis* outer spore coat proteins, but ExsF and ExsK are specific to *B. anthracis* and other members of the *Bacillus cereus* group.

INTRODUCTION

Bacillus anthracis, the aetiological agent of anthrax, is a Gram-positive, aerobic or facultative anaerobic spore-forming bacterium. The outermost layer of the spores from members of the *Bacillus cereus* group of micro-organisms, including *B. anthracis*, is the exosporium. This loose-fitting, balloon-like structure consists of two layers (Gerhardt & Ribi, 1964): a basal layer which has a hexagonally ordered crystal lattice structure (Gerhardt & Ribi, 1964; Beaman *et al.*, 1971) and a peripheral layer consisting of a nap of fine filaments termed the hairy nap (Roth & Williams, 1963; Gerhardt & Ribi, 1964; Hachisuka *et al.*, 1966; Moberly *et al.*, 1966; Kramer & Roth, 1968; Sylvestre *et al.*, 2003).

Much of the work published on the fine structure and composition of the exosporium to date has focused upon *B. cereus* (Gerhardt & Ribi, 1964; Matz *et al.*, 1970; Beaman *et al.*, 1971; Charlton *et al.*, 1999). This layer is chemically complex, consisting of protein, amino and neutral polysaccharides, lipids and ash (Matz *et al.*, 1970). *B. cereus* exosporium antigens first appear at the engulfment stage (stage III) of sporulation (DesRosier & Lara, 1984). *Bacillus thuringiensis* (Garcia-Patrone & Tandecarz, 1995), *B. cereus* (Charlton *et al.*, 1999) and *B. anthracis* (Sylvestre *et al.*, 2002) spores contain specific glycoproteins in their surface layers. The *B. anthracis* collagen-like spore surface glycoprotein (BclA) reported by Sylvestre *et al.* (2002) is a structural component of the filaments of the hairy nap and is highly immunogenic. Several of the proteins from the exosporium of *B. cereus* (Todd *et al.*, 2003) and *B. anthracis* (Steichen *et al.*, 2003) have been described, but the overall protein composition, structure and function of the exosporium have yet to be elucidated fully. The exosporium may play a role in the interaction of the spore with the infected host. For example, there may be an association between the exosporium of *B. anthracis* spores and the macrophage (Dixon *et al.*, 2000; Guidi-Rontani *et al.*, 2001), and exosporially located components, like the alanine racemase and inosine hydrolase reported here, may influence spore germination within the macrophage.

This study has identified several novel proteins tightly associated with the exosporium of *B. anthracis* that are likely

Abbreviation: DE-MALDI-TOF-MS, delayed extraction-matrix assisted laser desorption ionization-time of flight mass spectrometry.

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to represent integral exosporium proteins, as they are retained after salt and detergent washes.

METHODS

Bacterial strains, growth conditions and media. The *Bacillus anthracis* strains used were wild-type Ames, a pXO2-cured Ames derivative and UM23Cl2, a double-cured derivative of the Sterne strain which is not congenic with Ames. The wild-type Ames and pXO2-cured Ames derivative were handled in Advisory Committee on Dangerous Pathogens (ACDP) 3 containment, both as vegetative cells and as spores, whereas UM23Cl2 was grown at ACDP 2. The strains were grown routinely on nutrient agar (28 g Oxoid nutrient agar l^{-1}, pH 7·4), blood agar (40 g Oxoid blood agar base no. 2 l^{-1}; sterilized at 121 °C for 15 min, cooled to 50 °C and 50 ml sterile defibrinated horse blood added) or L-agar (10 g Difco tryptone peptone l^{-1}; 5 g Difco yeast extract l^{-1}; 20 g Difco Bacto agar l^{-1}; 5 g NaCl l^{-1}; pH 7·2) or in nutrient broth (13 g Oxoid Nutrient broth l^{-1}) or L-broth (10 g Difco tryptone peptone l^{-1}; 5 g Difco yeast extract l^{-1}; 5 g NaCl l^{-1}; pH 7·2) at 37 °C.

Spore preparation. Spores of *B. anthracis* were prepared on solid New Sporulation medium (3·0 g Difco Tryptone l^{-1}; 6·0 g Oxoid bacteriological peptone l^{-1}; 3·0 g Oxoid yeast extract l^{-1}; 1·5 g Oxoid Lab Lemco l^{-1}; 1 ml 0·1 % $MnCl_2.4H_2O$; 25 g Difco Bacto agar l^{-1}) at 37 °C, until the cultures contained more than 95 % free spores. Spores were harvested by centrifugation at 10 000 ***g*** for 10 min, and washed approximately 10 times in ice-cold sterile distilled water to remove contaminating vegetative cells and debris. Microscopic examination established that spore preparations contained less than 5 % vegetative cells and debris. The washed spore preparations were stored at −20 °C for 1 week to 30 months.

Exosporium removal and purification. Spores were defrosted and centrifuged at 10 000 ***g*** for 10 min at 4 °C. Prior to sonication, pellets were resuspended to between approximately 5×10^9 and $2{\cdot}5 \times 10^{10}$ spores ml^{-1} in 50 mM Tris/HCl, 0·5 mM EDTA buffer (pH 7·5). All subsequent manipulations were at 4 °C. To fragment and release exosporium material, spores were sonicated (amplitude 12 μm) using a Sanyo Soniprep 150 ultrasonic disintegrator, for seven 1 min bursts, each separated by 2 min cooling on ice. Exosporium fragments were separated from spores by pelleting the latter at 9000 ***g*** for 5 min. The spore pellet(s) were washed once in 50 mM Tris/HCl, 0·5 mM EDTA (pH 7·5) and the exosporium-containing supernatants were pooled, then centrifuged again (10 000 ***g***, 15 min) to pellet the remaining spores.

For all isolates of *B. anthracis*, any residual endospores in the exosporium-containing supernatant were removed by filtration through 0·45 and/or 0·2 μm low-protein-binding filters (Acrodisc, Gelman Sciences). The filtrate, containing exosporium, was subjected to a sterility check by inoculating 10 vols nutrient or L-broth with 10 % of the exosporium preparation and incubating at 37 °C with shaking (200 r.p.m.) for 7 days. After centrifugation to concentrate any cells, the incubated material was plated on L-agar, in parallel with the appropriate controls. The plates were incubated at 37 °C for 7 days and absence of growth of any colonies permitted clearance for use at a lower level of containment. Once confirmed as sterile, the exosporium-containing filtrate was concentrated approximately 15- to 50-fold using an Amicon ultrafiltration cell fitted with a polyethersulfone membrane (Millipore, NWWL 10 000) or by pelleting in an ultracentrifuge at 184 000 ***g*** for 1 h at 4 °C.

Salt and detergent washing of exosporium. The series of washes used on the exosporium was modified and simplified from a method for purifying *B. subtilis* spore coat fractions described by Nicholson & Setlow (1990). The technique was optimized using *B. anthracis* UM23Cl2 exosporium and subsequently applied to exosporium from the ACDP 3 strains of *B. anthracis*. The exosporium was washed with 1 M NaCl, followed by one or two washes in TEP buffer (50 mM Tris/HCl pH 7·2, 10 mM EDTA, 2 mM PMSF) containing 0·1 % (w/v) SDS. To remove the SDS, a final wash was in TEP buffer alone. Each wash involved resuspension of the purified exosporium followed by centrifugation in a Hettich microcentrifuge at 31 870 ***g*** for 40 min.

Protein determination. Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the manufacturer's instructions.

Alkali extraction of spore proteins. Alkali extraction was carried out on washed *B. anthracis* UM23Cl2 spores, as detailed by Sylvestre *et al.* (2002). Spores were incubated in alkaline reducing buffer without SDS (50 mM Tris/HCl, pH 10, 8 M urea and 2 % β-mercaptoethanol) for 15 min or 1 h, and the spores removed by centrifugation.

Electron microscopy. The samples were diluted in sterile distilled water, placed on Formvar-coated grids and examined after negative staining in 1 % (w/v) phosphotungstic acid in phosphate buffer (pH 7·2). After 15–30 s the excess phosphotungstic acid was withdrawn using filter paper. Samples were examined immediately using a Philips CM12 transmission electron microscope at an accelerating voltage of 60 kV.

Gel electrophoresis. Samples were mixed with an equal volume of sample buffer (125 mM Tris/HCl, pH 6·8; 20 %, v/v, glycerol; 4 %, w/v, SDS; 5 %, v/v, β-mercaptoethanol; 0·0005 %, w/v, bromophenol blue) and boiled for 5 min, then held on ice for 2–10 min and centrifuged briefly (30 s at 13 000 ***g***) to remove any insoluble material. The proteins were separated by SDS-PAGE in Tris-glycine buffer (24 mM Tris, 192 mM glycine and 0·1 %, w/v, SDS; Laemmli, 1970) at 125 V using pre-cast gels (Invitrogen), or on NuPAGE Bistris pre-cast gels in MES buffer (Invitrogen). To separate low-molecular-mass proteins, samples were boiled in Tricine gel sample buffer (900 mM Tris/HCl, pH 8·45; 24 %, v/v, glycerol; 8 %, w/v, SDS; 0·015 %, w/v, Coomassie blue G; 0·0005 %, w/v, phenol red; 5 %, v/v, β-mercaptoethanol) and separated on precast 16 % Tris-Tricine gels (Invitrogen) at 125 V using the Tricine running buffer (100 mM Tris; 100 mM Tricine; 0·1 %, w/v, SDS). Gels were stained with Colloidal Blue stain (Invitrogen) according to the manufacturer's instructions. For molecular mass determination, SigmaMarker molecular mass standards were used on both Tris-glycine and Tricine gels.

N-terminal sequencing. Proteins separated by SDS-PAGE were electrophoretically transferred onto PVDF membranes, using 10 mM CAPS transfer buffer (pH 11·0) with 10 % (v/v) methanol. The membranes were stained with 0·1 % (w/v) Coomassie 250 in 40 % (v/v) methanol/1 % (v/v) acetic acid, destained with 50 % (v/v) methanol and air-dried. N-terminal sequences were determined by automated Edman degradation in an Applied Biosystems gas-phase sequencer.

In-gel digestion and mass spectrometric analysis of protein samples. Bands identified for analysis from the SDS-PAGE gels were excised, reduced/carboxymethylated and subjected to in-gel tryptic digestion. The resulting peptides were extracted from the gel and purified by HPLC using a C_{18} cartridge. The peptide-containing fractions were collected and analysed by delayed extraction-matrix assisted laser desorption ionization-time of flight mass spectrometry (DE-MALDI-TOF-MS) and some peptides were subjected to sequencing using nanospray-MS/MS. All mass spectrometric analyses were performed at M-Scan Ltd, Wokingham, UK.

Sequence data. Sequences from protein bands were used to search preliminary sequence data for *B. anthracis* obtained from the

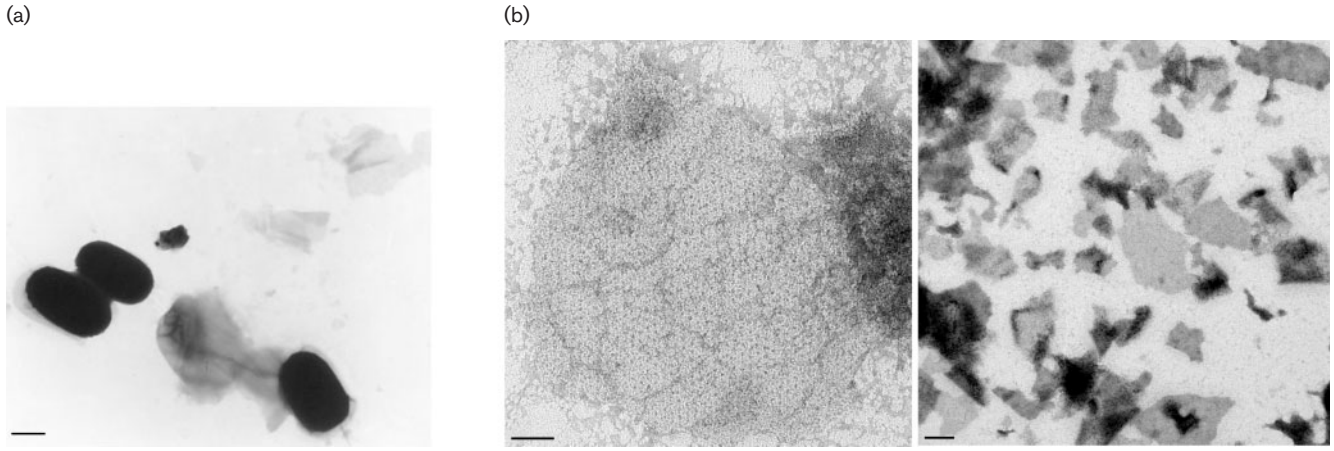

Fig. 1. Transmission electron micrographs of *B. anthracis* spores and exosporium. Negatively stained samples of (a) *B. anthracis* UM23Cl2 spores sonicated for 6 min at 12 μm (bar: 0·5 μm), and (b) filtered, salt-washed exosporium from sonicated *B. anthracis* wild-type Ames spores (bars: left, 50 nm; right 0·2 μm).

Institute for Genomic Research (TIGR) website at http://www.tigr.org, using tBLASTN (Altschul *et al.*, 1997), and to search ORFs identified in preliminary data for *B. cereus* ATCC 14579 from http://www.integratedgenomics.com. The wild-type Ames strain examined during this study was the parental strain for the double-cured Ames strain used for genome sequencing (Read *et al.*, 2003).

RESULTS AND DISCUSSION

Exosporium preparation

Exosporium was isolated from fully virulent *B. anthracis* Ames, from a derivative lacking pXO2 and from a variant of the Sterne strain, UM23CL2, lacking both pXO1 and pXO2. Because of the restrictions of working in ACDP 3 containment, exosporium was released using sonication rather than by a French press procedure. Sonication of spores for 6 or 7 min using the Sanyo Soniprep 150 ultrasonic disintegrator caused partial fragmentation of the exosporium without disruption of the spore (Fig. 1a). Some exosporium material was left attached to the spores. Whole spores were removed by low-speed centrifugation, the supernatant was filtered (0·45 μm or 0·2 μm) to remove all remaining live spores, and the exosporium fragments were concentrated. Small fragments with hexagonal periodicity were evident by electron microscopy (Fig. 1b).

Effects of washing the exosporium

A large number of protein bands from concentrated wild-type Ames exosporium were separated by SDS-PAGE (Fig. 2a, lane 2; Fig. 2b, lane 1). The supernatant from the

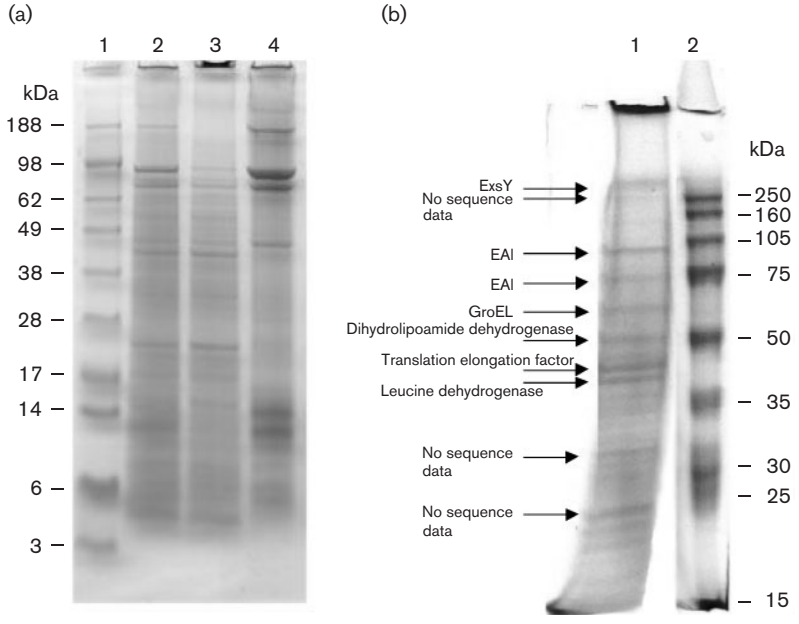

Fig. 2. Comparison of the protein profiles of unwashed and salt/detergent-washed *B. anthracis* exosporium. (a) Proteins in exosporium extracts of *B. anthracis* wild-type Ames were separated by SDS-PAGE on a 10% NuPAGE Bistris gel, and stained with Colloidal blue. Lane 2, unwashed exosporium; lane 3, proteins in the supernatant of the centrifuged, unwashed, exosporium extract; lane 4, fully washed exosporium extract. (b) Proteins in an unwashed *B. anthracis* Ames exosporium preparation (lane 1) were separated by SDS-PAGE on a 10% Tris-glycine gel and stained with Colloidal blue stain. Selected bands, indicated by arrows, were subjected to DE-MALDI-TOF-MS, peptide sequences were obtained by nanospray-MS/MS, and unequivocal protein assignments were made.

first centrifugation to concentrate the material after filtration (Fig. 2a, lane 3) contained many of the same bands. The exosporium preparation was washed successively with salt (1 M NaCl) and detergent (0·1 % SDS) to remove loosely adsorbed proteins. The exosporium protein profile after these washing procedures (Fig. 2a, lane 4) was enriched for the proteins in the original extract (lane 2) that were less strongly represented in the supernatant fraction in lane 3.

Identification of proteins in unwashed exosporium

Several protein bands from SDS-PAGE gels of unwashed exosporium preparations were identified by N-terminal sequencing and/or nanospray MS/MS sequencing following DE-MALDI-TOF-MS, which provides information on N-terminal, mid-chain and C-terminal sequence with equal probability. Peptide sequences were searched against predicted ORFs of *B. anthracis* (provided by T. Read, TIGR). An example of a profile used for analysis is in Fig. 2(b), and Table 1 lists the proteins identified.

B. anthracis vegetative cells produce a proteinaceous paracrystalline sheath termed the S-layer which completely covers the cell surface. The S-layer protein EA1 is expressed during the stationary phase of growth (Mignot *et al.*, 2002). EA1 was frequently detected in protein profiles, from both unwashed and washed *B. anthracis* exosporium, as multiple bands of about 98 and 75 kDa (Fig. 2b, Table 1), by MALDI-TOF-MS. These exosporium preparations had not been purified on density gradients, and therefore could contain contaminating S-layer lattice material (Mesnage *et al.*, 1997; Mignot *et al.*, 2002). The extent of cross-contamination with EA1 varied in different preparations.

Three of the nine other proteins identified in unwashed exosporium (alanine racemase, CotY and ExsY) were also identified in washed exosporium (Fig. 3). GroEL has already been reported as an exosporium-adsorbed protein in *B. cereus*, removable by salt washing (Charlton *et al.*, 1999; Todd *et al.*, 2003). The Zn-dependent metalloprotease immune inhibitor A (InhA), a major component of unwashed exosporium preparations of *B. cereus*, was not detectable in *B. anthracis* exosporium.

An exhaustive study of the many other bands in the unwashed material was not undertaken; instead, the exosporium preparations were washed more stringently, to identify tightly bound or integral proteins.

Identification of proteins in the washed exosporium

Salt- and detergent-washed exosporium preparations from all three strains examined showed a generally reproducible pattern of proteins on SDS-PAGE on a 16 % acrylamide, Tris-Tricine-buffered gel (Fig. 3), except for the variable EA1 S-layer proteins (as in lane 3, bands 1, 2 and 3).

A number of bands were extracted and mass spectrometry (MS/MS sequencing of tryptic peptides) provided internal sequences for all bands tested except the largest (> 200 kDa; Fig. 3). Several protein components were identified – their amino acid sequences, predicted from the *B. anthracis* genome sequence, are shown in Table 2.

Table 1. Proteins identified in unwashed exosporium from *B. anthracis* spores

Gene identification (Protein)	Apparent mol. mass (kDa)	Predicted mol. mass (kDa)	Predicted pI	N-terminal sequence	Peptide sequences
BA0252 Alanine racemase	48	43·7	5·51	MEEAPFYRDT*	
BA0887 S-layer protein EA1	98	91·4	5·70		SDPENLE†, NLPVD†
BA0887 S-layer protein EA1	75	91·4	5·70		VEIVGETI†, YGDPF†
BA0267 GroEL	61	57·4	4·79		ELEDAFENM†, VGNDGVITIEESK†
BA1629 Cold-shock protein (CspB-1)	7·3	7·2	4·60	MQNGKVKQFNSEK†	
BA1238 CotY	17·8	16·8	4·71	SLNXNEDHHHHDXDFNXV*	
BA1234 ExsY	16·1	16·1	4·95	SXNENKHHGSXHNVVDEVKF*	
BA1234 ExsY	> 250	16·1	4·95		SVDDDSCAVLR†, PFEAFA†
BA2332	14·4	14·4	5·24	MFFAKLRGRNEVPPVETDARG*	
BA4181 Dihydrolipoamide dehydrogenase (PdhD)	50	49·5	5·26		AYFVDAN†
BA0108 Translation elongation factor Tu	43	42·9	4·93		DQIDAA†
BA4387 Leucine dehydrogenase	40	39·9	5·13		FGTDXXEGK†

Sequences identified from: *pXO2-cured Ames derivative exosporium; †wild-type Ames exosporium.

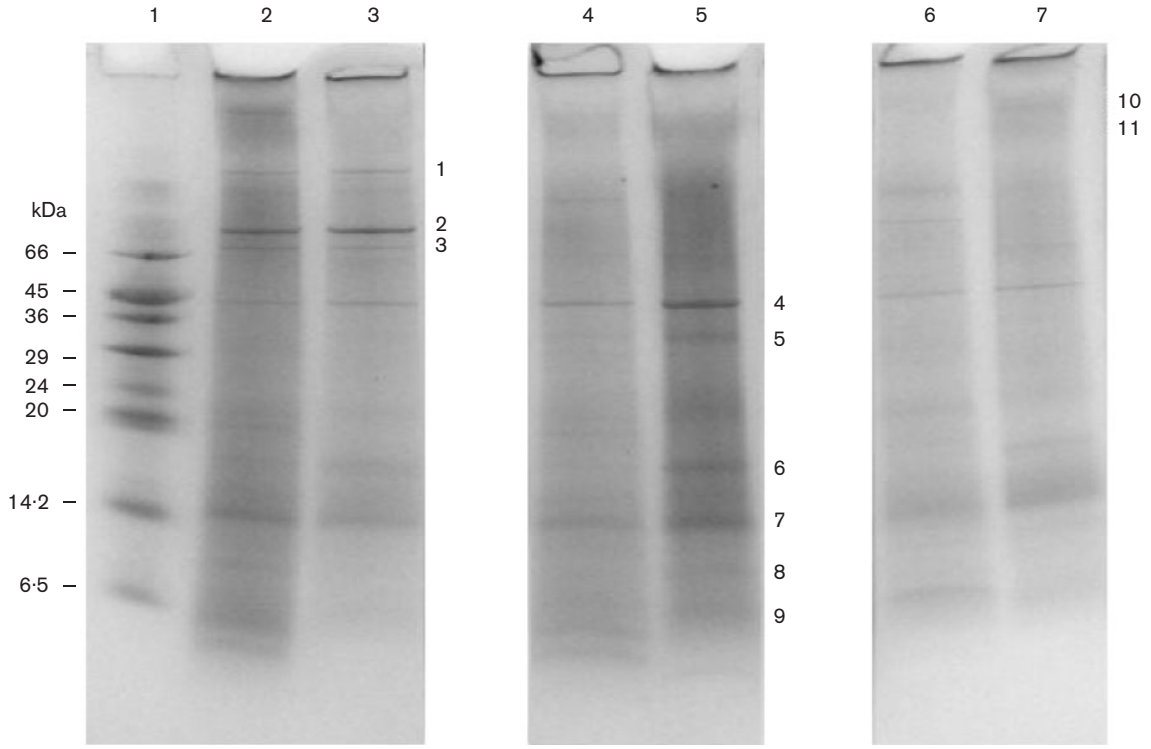

Fig. 3. Identification of selected proteins from washed *B. anthracis* exosporium. Proteins from exosporium extracts were separated on a 16 % Tricine gel, and stained with Colloidal blue. Lane 1, SigmaMarker low-range molecular mass marker; lanes 2 and 3, *B. anthracis* UM23Cl2, unwashed and washed respectively; lanes 4 and 5, *B. anthracis* pXO1$^+$/pXO2$^-$ Ames, unwashed and washed respectively; lanes 6 and 7, *B. anthracis* wild-type Ames, unwashed and washed respectively. Selected bands (numbered) were subjected to DE-MALDI-TOF-MS and a number of peptides were identified by nanospray-MS/MS.

The strong band with apparent molecular mass of 43 kDa present in both unwashed and washed exosporium was identified from both MS sequencing (Fig. 3, band 4; Table 2) and direct N-terminal sequencing (Table 1) as the alanine racemase homologue BA0252 (50 % amino acid identity to the *B. subtilis dal* gene product). A second alanine racemase homologue encoded in the *B. anthracis* genome sequence has a very different N-terminal amino acid sequence (only 10 out of 21 identities with the observed N-terminus). The band at 33 kDa (Fig. 3, band 5) (Table 2) contains a homologue of inosine-uridine-preferring nucleoside hydrolases (30 % amino acid identity with the paradigm protozoal enzyme of Gopaul *et al.* (1996). These two enzymes have also been shown to be present in washed *B. cereus* UM20.1 exosporium (Todd *et al.*, 2003). Alanine racemase has been detected in exosporium of *B. anthracis* (Steichen *et al.*, 2003) and inosine hydrolase in alkali-extracted material from *B. anthracis* spores (Lai *et al.*, 2003). Both proteins represent enzymes of potential importance to the spore. Both alanine and inosine are potential germinants for *B. anthracis*, although additional components are required for the inosine response (Hills, 1949; Ireland & Hanna, 2002; Titball & Manchee, 1987). These enzymes may be involved in metabolism of germinants and presumably moderate the rate of spore germination.

The band at 17 kDa (Fig. 3, band 6) contained sequences that correspond to a protein encoded by *B. anthracis* and very recently described and designated BxpB by Steichen *et al.* (2003). The predicted molecular mass of 17 kDa corresponds to that observed experimentally. The equivalent protein detected in *B. cereus* exosporium shares 97 % aa identity and has been designated ExsF (Todd *et al.*, 2003). A related paralogue is also encoded in the *B. anthracis* genome sequence, but the sequence PVIND is the only one found that could be from this paralogue, and it is also present in BxpB/ExsF.

The band at 14 kDa (Fig. 3, band 7) contains sequences that have been identified as derived from at least two, and possibly three, separate proteins. One is a homologue of (30 % identity to) *B. subtilis* spore coat protein CotB (Table 2), of predicted size 19·4 kDa. CotB (Donovan *et al.*, 1987; Driks, 1999) is exposed on the spore surface of *B. subtilis*, and has even been used as an anchor for non-native antigens (Isticato *et al.*, 2001). Its location in *B. anthracis* will be determined using immunological

Table 2. Proteins identified in washed exosporium from *B. anthracis* spores

Gene identification* (Protein)	Apparent mol. mass (kDa)	Predicted mol. mass (kDa)	Predicted pI	Peptide sequences†
BA0252 Alanine racemase (Dal)	43	43·6	5·51	**HTANS (Band 4); VLVNGK (Band 4)** MEEAPFYRDTWVEVDLDAIYNNVTHIKEFIPSDVEIFAVVKGN- AYGHDYVPVAKIALEAGATRLAVAFLDEALVLRRAGITAPILV- LGPSPPRDINVAAENDVALTVFQKEWVDEAIKLWDGSSTMK- YHINFDSGMGRIGIRERKELKGFLKSLEGAPFLELEGVYTHFA- TADEVETSYFDKQYNTFLEQLSWLKEFGVDPKFVHTANSAA- TLRFQGITFNAVRIGIAMYGLSPSVEIRPFLPFKLEPALSLHTK- VAHIKQVIKGDGISYNVTYRTKTEEWIATVAIGYADGWLRR- LQGFEVLVNGKRVPIVGRVTMDQFMIHLPCEVPLGTKVTLI- GRQGDEYISATEVAEYSGTINYEIITTISFRVPRIFIRNGKVVEV- INYLNDI
BA2888 Inosine-preferring nucleoside hydrolase	33	36·3	5·05	**VTENVYFLER (Band 5); ISFINDD (Band 5)** MSIVNKKIIFFGDFGIDDAVALIYANKTCKLDILGIVAEYGNVS- REIVTENVYFLERYYATEVKIIEGAARPMTAEEPLFFPEIHGEH- GLGPIIPPDLRICKRENFCELIKLIEPCPEDIIIVATGRLTTLATL- FLLYPNVMDKVCSYYIMGGAFLFPGNVTPVSEANFYGDPIAA- NIVMKYAKNAHIYPLNVTQEALITPEMVDIINKEGTGQAKLI- KPMIDFYYENFYKKEYPGIAGSPIHDLLPFISFINDDIFEYKKSA- VWISTTNDVTRGQSVADFRKIAEPTRFDDRPVQKIAVGFNYP- AFKEEFMRTILKPDCP
BA1237 Hypothetical protein (BxpB/ExsF)	17	17·3	4·43	**MFSSD (Band 11); TISLDNS (Band 6); PVELI (Band 6)** MFSSDCEFTKIDCEAKPASTLPAFGFAFNASAPQFASLFTPLLLP- SVSPNPNITVPVINDTVSVGDGIRILRAGIYQISYTLTISLDNSP- VAPEAGRFFLSLGTPANIIPGSGTAVRSNVIGTGEVDVSSGVIL- INLNPGDLIRIVPVELIGTVDIRAAALTVAQIS
BA1238 Spore coat protein Z (CotZ) CotY	14 and 6·5	16·8	4·71	**FEAFAP (Band 7, 8 & 11); SANLT (Band 9); SIDDDD (Band 9)** MSCNCNEDHHHHDCDFNCVSNVVRFIHELQECATTTCGSG- CEVPFLGAHNSASVANTRPFILYTKAGAPFEAFAPSANLTSCR- SPIFRVESIDDDDCAVLRVLSVVLGDTSPVPPTDDPICTFLAV- PNARLISTNTCLTVDLSCFCAIQCLRDVTI
BA1234 Spore coat protein Z (CotZ) ExsY	14 and 10	16·1	4·95	**FEAFAP (Band 7, 8 & 11)** MSCNENKHHGSSHCVVDVVKFINELQDCSTTTCGSGCEIPFL- GAHNTASVANTRPFILYTKAGAPFEAFAPSANLTSCRSPIFRV- ESVDDDSCAVLRVLSVVLGDSSPVPPTDDPICTFLAVPNARL- VSTSTCITVDLSCFCAIQCLRDVTI
BA0355 CotB homologue	14	19·4	5·31	**ISVCPD (Band 7)** MSLFHCDFLKDLIGSFVRVNRGGPESQRGTIISVCPDYFVLQNE- KGELYYYQLSHLKSITKSAKECGSSDCEWEDCACAEDFEALLE- SFKYCWVKINRGGPEKVEGILQDVSCDFVTLIVKEEIILIAIKHI- KSVNYNALACGESDESDDSSSKESSNNSGRARAQRQSSRGR
BA2554 Hypothetical protein ExsK	10	12·0	7·96	**NYAGTVS (Band 8)** MGSRYSNSRKKCSCKQCSCKQDDCWDVFEECKKEHEEQNKA- CDCCCVQGIRDELRKLVNRSVRITTGSNNYAGTVSSVTCDVV- KLANSAGVVTVIISVCKIEAIEALLT

*ORF numbers from TIGR genome sequence.

†Band numbering alongside peptide sequences correspond to the numbered bands in Fig. 3.

techniques. The other sequences from the 14 kDa band (FEAFAP and SANLT) are present in the sequences of two proteins, ExsY and CotY, as named in Todd *et al.* (2003). These are paralogues (84 % identity) and are more distant homologues of the *B. subtilis* CotZ and CotY proteins, which are present in the insoluble outer layers of the *B. subtilis* spore coat (Driks, 1999). ExsY is required for exosporium assembly (M. Johnson, S. J. Todd & A. Moir, unpublished).

The *cotY* gene forms part of a complex operon in *B. subtilis* which consists of five genes, *cotVWXYZ*. It is hypothesized that the complex transcriptional pattern of this cluster is necessary to maintain an optimal amount of each protein for correct assembly of the spore coat in *B. subtilis* (Zhang *et al.*, 1993). There are no ORFs homologous to CotV, W or X proteins in the *B. anthracis* genome.

Lai *et al.* (2003) also reported the presence of this CotB homologue and proteins similar to ExsY or CotY in spore coat extracts prepared by boiling *B. anthracis* spores in SDS-PAGE loading buffer for 10 min.

The band at 10 kDa (Fig. 3, band 8) contains peptides from a predicted protein, which we have named ExsK. The ExsK protein has not previously been described as a coat or exosporium component. There is a significantly divergent homologue encoded in the *B. cereus* ATCC 14579 genome sequence with 64 % aa identity. Direct comparison of ExsK with *B. subtilis* proteins by FASTA gives the highest, though not significant score ($e=0{\cdot}12$) as the *B. subtilis* outer coat protein CotX. ExsK has no valid homologues in *B. subtilis*, but has some similarity in composition near the N-terminus (a very charged region at residues 29–37, preceding a cysteine-rich region) to the equivalent region of CotX.

Peptide FEAFAP was also present in the 10 kDa band (Fig. 3, band 8), suggesting that either or both ExsY and CotY sequences are present. The band at about 6·5 kDa (Fig. 3, band 9) contained a peptide sequence (SIDDDD) specific to CotY. Presumably these proteins in the 10 and 6·5 kDa positions are partial degradation/processing products, as the predicted size of the intact proteins would be 16–17 kDa.

Peptide sequences were obtained from the second-largest band (Fig. 3, band 11) (MFSSD and FEAFAP), demonstrating the presence of BxpB (ExsF) and either or both of ExsY and CotY in this band. These proteins are therefore present in the gel in high-molecular-mass complexes, as well as in a monomeric form (BxpB/ExsF) or in a proteolytically cleaved form (ExsY/CotY).

Alkali extraction of proteins from spores

Spores of *B. anthracis* UM23Cl2 were incubated with alkaline reducing buffer without SDS, as used by Sylvestre *et al.* (2002) to extract a fraction containing BclA, a major exosporium glycoprotein. Large bands in an SDS-PAGE protein profile of the extracted proteins (Fig. 4) were subjected to MS/MS and N-terminal sequencing. The diffuse multiple band (>250 kDa) in the 15 min extract (Fig. 4, lane 2) contained peptides from ExsY (SVDDDS) and ExsF (MFSSD) and yielded the N-terminal sequence for glycoprotein BclA (AFDPNLVGPT). Specific staining confirmed the presence of glycoprotein in this region of the gel (data not shown). The presence of BclA at this position in unwashed and washed exosporium of *B. anthracis* exosporium was also demonstrated by immunoblotting with specific antibodies (data not shown). On incubation for

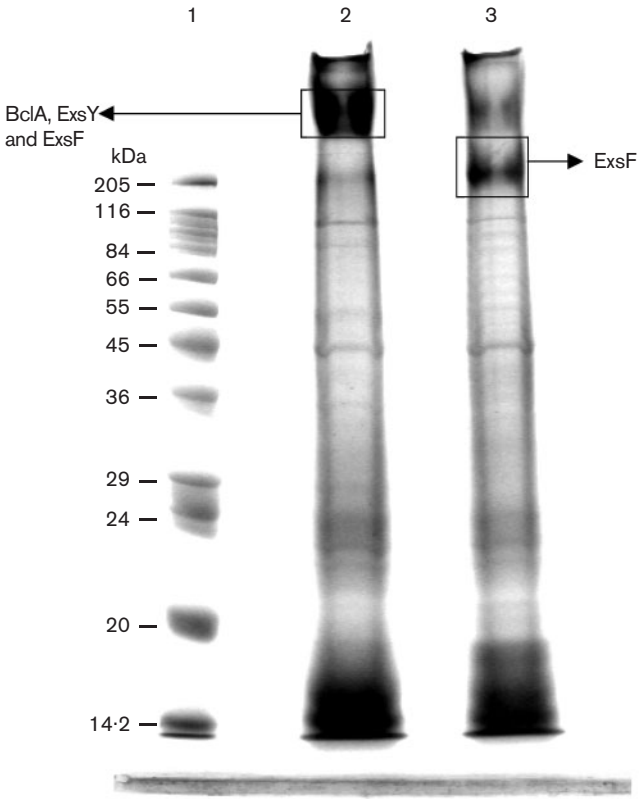

Fig. 4. Alkali-extracted proteins from *B. anthracis* UM23Cl2 spores. Spores of *B. anthracis* UM23Cl2 were incubated in the alkaline extraction buffer of Sylvestre *et al.* (2002) and the extracted proteins were separated on a 12 % Tris-glycine gel. Extraction for 15 min (lane 2) and 1 h (lane 3). SigmaMarker broad-range molecular mass marker is in lane 1.

60 min in alkaline extraction buffer, a smaller band at 205 kDa increased in intensity (Fig. 4, lane 3), indicating some dissociation; peptides PVIND and PVELI from ExsF were both identified in this band. The difficulties in dissociating proteins ExsY, CotY and ExsF to their monomeric sizes suggest that these proteins in the spore are involved in relatively stable multimeric complexes, along with the glycoprotein BclA, in the exosporium.

Proteins of the exosporium of *B. anthracis*

As only selected bands were examined, from a one-dimensional gel of limited resolution, and as some material did not enter the gel, only a small proportion of the total proteins in the exosporium have been identified in this analysis. Nevertheless, this work demonstrates the presence of four proteins in salt-washed exosporium that have not previously been reported – ExsK, CotB, ExsY and CotY. The latter two have been detected in multimeric complexes, with ExsF and BclA, as well as at the monomeric size, or even smaller. ExsK is a novel protein, of unusual composition – the first half of the protein contains 20 % charged residues

and 10 % cysteine residues; the second half, which is less conserved, is more hydrophobic in nature (Table 2).

Of the exosporium proteins identified by Steichen *et al.* (2003), BclA and BxpB (ExsF) are also reported here, but their BxpC protein has not been sequenced in this study. Of the proteins identified by Todd *et al.* (2003) in washed *B. cereus* ATCC 10876 exosporium that are also encoded in the *B. anthracis* genome, those designated ExsB, D, E, G and CotE have as yet not been identified directly in *B. anthracis* exosporium, although ExsF is reported here. As sequence data have only been obtained for relatively few of the proteins separated from washed *B. anthracis* exosporium, it may be that some or all of these proteins remain to be identified.

This is believed to be the only published analysis of the exosporium of the fully virulent, double-plasmid-containing Ames strain of *B. anthracis*. There are no obvious differences in gross exosporium profile between the wild-type and cured strains, and all of the proteins identified here are encoded chromosomally. Therefore the cured strains represent good models for studying the exosporium from spores of *B. anthracis*.

In addition to the demonstrated salt- and detergent-resistant components, unwashed exosporium contains other proteins, some described here, that may contribute to the *in vivo* activities of the exosporium. Some of these proteins may not be exosporium-bound, but may be located between the exosporium and the rest of the spore, and associate during isolation. By no means all of the exosporium proteins, whether loosely associated/adsorbed, or tightly bound, and salt- and detergent-resistant, have yet been identified. The role of the exosporium of *B. anthracis*, and the contribution to its structure of the CotB, ExsF, ExsY, CotY and ExsK proteins defined here, are issues that remain to be addressed in future experiments.

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