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(54) Title: IMMUNOGENIC PROTEIN AND USES THEREOF

(57) Abstract: This invention relates to outer layer proteins of *Bacillus anthracis* for use in producing an immunogenic response, which can then be applied in the detection of *B.anthraxis*, and in the preparation of vaccines against *B.anthraxis* infection. Antibodies which recognise these proteins and methods for the detection of *B.anthraxis* are also described and claimed.



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Immunogenic Protein and Uses thereof

5 The present invention relates to outer layer proteins of
Bacillus anthracis for use in producing an immunogenic response,
which can then be applied in the detection of *B. anthracis*, and
in the preparation of vaccines against *B. anthracis* infection.
In addition, the invention provides antibodies which
10 specifically recognise these proteins, and certain novel
proteins themselves, together with nucleic acids encoding them.

Bacillus anthracis, the aetiological agent of anthrax, is a Gram
positive, aerobic or facultative anaerobic spore forming
15 bacterium. The outer most integument 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 and Ribí, 1964); a
basal layer which has a hexagonally ordered crystal lattice
20 structure (Gerhardt and Ribí, 1964; Beaman et al., 1971) and a
second peripheral layer consisting of a nap of fine filaments
termed the hairy nap (Roth and Williams, 1963; Gerhardt and
Ribí, 1964; Hachisuka et al., 1966; Moberly et al., 1966; Kramer
and Roth, 1968).

25 Much of the work published on the fine structure and composition
of the exosporium to date has focused upon *B. cereus* (Gerhardt
and Ribí, 1964; Matz et al., 1970; Beaman et al., 1971; Charlton
et al., 1999). This layer is chemically complex, consisting of
30 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 and Lara,
1984). *B. thuringiensis* (Garcia-Patrone and Tandecarz, 1995),
B. cereus (Charlton et al., 1999) and *B. anthracis* (Sylvestre et
35 al., 2002) spores contain glycoproteins specific 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. However, the protein composition, structure and function of the exosporium have still 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.

The applicants have identified several proteins which are tightly associated with the exosporium of *B. anthracis* and which appear to represent integral exosporium proteins, as they are retained after salt and detergent washes.

According to the present invention, there is provided a protein obtainable from an outer layer of *B. anthracis* in a pre-vegetative state, which protein is either (a) retained in said layer after washing with a salt solution, followed by washing with detergent, and/or (b) retained in a supernatant of said layer which supernatant is obtained after having first removed the exosporium from said *B. anthracis* in a pre-vegetative state; wherein said protein has one or more regions specific to *B. anthracis*, or a variant of said protein, or a fragment of any of these, for use in producing an immunogenic response to *B. anthracis*.

In the context of the present invention, the expression "has one or more regions specific to *B. anthracis*" refers to any protein which can be used to positively differentiate the *Bacillus anthracis* species from another *Bacillus* species. Such proteins could include those proteins that are only present in *B.*

anthracis and are not identified in other *Bacillus* species. These proteins are known as proteins specific to *B. anthracis*. Alternatively such a protein could include those proteins which have one or more amino acid sequences with an identical or close
5 homology to a protein naturally present in *B. anthracis* but wherein the proteins are also sufficiently different from proteins naturally present in other *Bacillus* species to enable differentiation between the species. It is likely that such a protein has a marked difference in sequence identity to that
10 obtainable in a similar manner from another *Bacillus* species. For example such a protein may have less than 90% sequence identity, preferably less than 80% sequence identity and more preferably less than 70% sequence identity to any protein obtainable in a similar manner from those of another *Bacillus*
15 species. It is to be understood herein that such proteins also include variants of such proteins, or a fragment of any of these.

For the avoidance of doubt the term protein when used herein is
20 considered to embrace those compounds which are also known in the art as glycoproteins.

In the context of the present invention, the expression "outer layer" refers to any layers found in the surface region of *B. anthracis* including the spore coat layers and in particular the
25 exosporium.

In addition, the expression "variant" as used herein refers to sequences of amino acids which differ from the base sequence
30 from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are
35 where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions

will be possible without altering the biological activity of the polypeptide. Suitably variants will be at least 80% identical, preferably at least 90% identical, and more preferably at least 95% identical to the base sequence.

5

Identity in this instance can be judged for example using the algorithm of Lipman-Pearson, with Ktuple:2, gap penalty:4, Gap Length Penalty:12, standard PAM scoring matrix (Lipman, D.J. and Pearson, W.R., Rapid and Sensitive Protein Similarity Searches, 10 *Science*, 1985, vol. 227, 1435-1441).

The term "fragment thereof" refers to any portion of the given amino acid sequence which has the same activity as the complete amino acid sequence. Fragments will suitably comprise at least 15 5 and preferably at least 10 consecutive amino acids from the basic sequence, or combinations of these fragments.

Proteins of the invention are particularly useful for raising antibodies, which are useful in the detection of *B. anthracis*. 20 The use of proteins which are present in the spore state is helpful in that it allows for rapid detection of *B. anthracis*, without the need for extensive culturing of the cells.

In the context of the present invention, the proteins obtainable 25 as set out may either be structural proteins or proteins which are associated with the outer layers of the *B. anthracis* by adsorption or by some other means.

Proteins which are retained in the outer layers, and in particular are integral with the outer layers, are more readily 30 and reliably available for detection purposes. Furthermore, proteins that have one or more regions specific to *B. anthracis*, in particular those which have less than 90% identity to those of a further *Bacillus* species, for example *B. cereus*, will allow distinction between the species to be readily made.

35 In particular proteins which are structural or integral with the outer layers such as the exosporium, are most likely to give

rise to a strong immune response, which will be protective against infection by *B. anthracis*. Thus proteins as described above may be used in producing an immune response in a host animal, which response is protective against infection by *B. anthracis*.

In one embodiment of the invention, the protein is one that is retained in the outer layer, and in particular in the exosporium, after washing with salt solution, for example 1M NaCl, followed by washing with detergent. As discussed above, such proteins are believed to be structural proteins of the outer layer, or at least are integral with said layer. Such proteins can optionally be identified by first removing the exosporium layer and then washing as set out.

Examples of such proteins are proteins of SEQ ID NO 1, 2, 3, 4, 5, 6 or 7 as shown in Table 2. A particular example of such proteins is a protein of SEQ ID NO 6 that has less than 80% identity with any protein found in *B. cereus*.

Another particular examples is the protein of SEQ ID NO 7, which has been designated ExsK. The ExsK protein has not previously been described as a coat or exosporium component, although a homologue (64% identity) is encoded in *B. cereus* ATCC genome 14579. Thus this is novel protein and forms a particular aspect of the invention, together with variants having greater than 64% identity thereto, or immunogenic fragments of any of these.

In another embodiment of the invention, the protein is one that is retained in a supernatant of the outer layer, which supernatant is obtained after having first removed the exosporium from said *B. anthracis* in a pre-vegetative state. The exosporium can be removed by any means known to one skilled in the art. For example the exosporium can be removed by sonicating the sample, using a bead beater, using a French press or other well know methods. Proteins obtained in this manner

are not believed to be structural proteins of the outer layer, but rather to be proteins which are adsorbed onto the outer layers of said *B. anthracis*.

5 A particular protein which is obtainable from an outer layer of *B. anthracis* and has one or more regions specific to *B. anthracis*, in particular those which have less than 80% identity to any protein obtainable from *B. cereus* is the protein which is designated ExsL as defined in Table 1. Although this protein
10 does not appear to be retained in exosporium washed as described herein, it is present in the outer layers and has only 42% identity to a protein encoded by a gene in the *B. cereus* genome.

Proteins of the invention may be prepared using conventional
15 methods. Although they may be isolated from *B. anthracis*, it is preferable that they are expressed recombinantly. For this purpose, a nucleic acid encoding the proteins is incorporated into an expression vector or plasmid, which is then used to transform a host cell. The host cell may be a prokaryotic or
20 eukaryotic cell, but is preferably a prokaryotic cell such as *E. coli*. The codons utilised in the nucleic acid may be optimised for expression in the particular host cell.

Nucleic acids encoding novel proteins of the invention such as
25 SEQ ID NO 7, as well as vectors and cells containing these form a further aspect of the invention.

Once produced, the proteins of the invention can be used to produce binding moieties comprising an antibody or a binding
30 fragment thereof, which specifically recognises a protein according to any one of the preceding claims. These form yet a further aspect of the invention.

Antibodies or binding fragments thereof may be polyclonal or
35 monoclonal, which may be produced using conventional methods.

For instance, polyclonal antibodies may be generated by immunisation of an animal (such as a rabbit, rat, goat, horse, sheep etc) with immunogenic proteins or immunogenic subunits or fragments thereof, to raise antisera, from which antibodies may
5 be purified.

Monoclonal antibodies may be obtained by fusing spleen cells from an immunised animal with myeloma cells, and selecting hybridoma cells which secrete suitable antibodies.

10

Antibody binding fragments include $F(ab')_2$, $F(ab)_2$, Fab or Fab' fragments, as well as recombinant antibodies, such as single chain (sc) antibodies FV, VH or VK fragments, but they may also comprise deletion mutants of an antibody sequence. Acronyms
15 used here are well known in the art. They are suitably derived from polyclonal or monoclonal antibodies using conventional methods such as enzymatic digestion with enzymes such as papain or pepsin (to produce Fab and $F(ab')_2$ fragments respectively). Alternatively, they may be generated using conventional
20 recombinant DNA technology.

Once obtained, the binding moieties are useful in detecting *B. anthracis*. Thus the invention further provides a method for detecting the presence of *B. anthracis* which method comprises
25 contacting a sample suspected of containing *B. anthracis* cells with a binding moiety as described above, and detecting binding therebetween.

Detection methods used include conventional immunological
30 methods for example ELISA, surface plasmon resonance and the like.

The sample is suitably an environmental sample, suspected of containing *B. anthracis* spores. These do not have to be
35 cultured to form vegetative cells but can be used directly in the detection method.

Suitably the binding moiety is immobilised on a solid support, for example on an ELISA plate, but other forms of support, for example membranes such as those utilised in conventional "dip-stick" tests may also be employed.

Detection of a complex between a protein within a spore in the sample, and a binding moiety as described above can be detected using conventional methods, in particular immunological methods such as ELISA methods. Assay formats may take various forms including "sandwich" or "competitive" types.

In a typical sandwich assay, the binding moiety is immobilised on a support, such as an ELISA plate, where is it contacted with a sample suspected of containing anthrax spores. Where present, these spores will bind the binding moiety and so become immobilised in their turn. The support is then separated from the sample, for example by washing. The presence of spores on the support can then be detected by application of secondary antibodies or binding fragments thereof, which bind to the spore, and are detectable, for example because they are labelled for instance with a visible label such as a fluorescent label, or a radiolabel, but preferably that they can be developed to produce a visible signal. A particular example of a secondary antibody is an antibody or binding fragment, that carries an enzymatic label, such as horseradish peroxidase, which can then be utilised to produce a signal by addition of the enzyme substrate, using conventional ELISA methodology. Secondary antibodies used in this way may also comprise binding moieties in accordance with the invention.

In a particular competitive assay format, the binding moiety of the invention is immobilised on a support. In this instance, a protein which binds said binding moiety in competition to the spores is added to the sample prior to contact with the support. Any spores present within the sample will compete with this

protein for binding to the immobilised binding moiety. Thus, the absence of peptide on the support is indicative of the presence of spores in the sample.

5 In this case, the competing protein is suitably labelled so that it may be readily detected, for instance using a visible label such as a fluorescent or radiolabel. Alternatively, it may be detected using a secondary antibody or a binding fragment thereof, such as those discussed above in relation to sandwich
10 assays, which binds the protein.

The proteins of the invention may also have pharmaceutical application as vaccines, as they may produce a protective immune response in a host animal, such as a human to whom they are
15 administered. Methods of treatment in this way form yet a further aspect of the invention.

For pharmaceutical application, the proteins are suitably administered in the form of a pharmaceutical composition. Thus
20 the invention further provides a pharmaceutical composition comprising a protein as described above in combination with a pharmaceutically acceptable carrier.

Compositions of the invention may further comprise
25 pharmaceutically acceptable carriers or excipients as are well known in the art. They may be solid or liquid carriers depending upon the intended mode of administration.

Any desired mode of administration may be used to deliver the
30 exosporium proteins for the purposes of inducing immunological responses in human or animal recipients. Particularly, although not exclusively, compositions of the invention will be intended for parenteral, including intramuscular, subcutaneous, intradermal, intraperitoneal and intravenous administration, or
35 non parenteral including intranasal, inhalation, oral, buccal, epidermal, transcutaneous, ocular-topical, vaginal, rectal

administration. The exact means of administration can be readily determined by one skilled in the art and will depend upon factors such as the nature of the toxin being treated, and the nature of the patient. It is preferred that compositions of the invention are intended for oral, intravenous or intranasal administration, or for administration by inhalation.

Compositions for parenteral administration will suitably be in the form of sterile solutions or suspensions which may contain suitable adjuvants (for example Alhydrogel™) and / or other excipients. Immunomodulators, such as cytokines, chemokines, synthetic or natural molecules of bacterial origin or any other immunomodulatory materials may also be included. Alternatively, the outer spore surface protein(s) may be formulated into emulsions, biodegradable microspheres or liposomes or any other suitable delivery system.

It is envisaged that the outer spore surface proteins may be administered to vaccines by nonparenteral (intranasal, inhalational, oral, buccal, epidermal, transcutaneous, ocular-topical, vaginal, rectal) routes. For nonparenteral delivery, antigens may be formulated into biodegradable microspheres or liposomes or any other suitable delivery system. Adjuvants, for example those derived from enterotoxins, may be included in the composition. Cytokines, chemokines, synthetic or natural molecules of bacterial origin or any other immunomodulatory molecule(s) may also be included to amplify / modify the immunological response following nonparenteral delivery of the outer spore surface proteins. Oral compositions may be in the form of tablets, lozenges, hard or soft capsules, aqueous or oily suspensions, emulsions, dispersible powders or granules, syrups or elixirs.

Compositions for the delivery of the formulation into the respiratory tract may be in the form of a finely divided dry powder.

Compositions of the invention may comprise other components such as preservative agents, inert diluents, granulating and disintegrating agents, binding agents, lubricating agents, 5 anti-oxidants as well as colouring, sweetening or flavouring agents, depending upon the nature of the composition.

Compositions for administration by inhalation may be in the form of a conventional pressurised aerosol arranged to dispense the 10 active ingredient either as an aerosol containing finely divided solid or liquid droplets. Conventional aerosol propellants such as volatile fluorinated hydrocarbons or hydrocarbons may be used and the aerosol device is conveniently arranged to dispense a metered quantity of active ingredient.

15 The relative amounts of pharmaceutically acceptable carrier to the protein in a formulation will vary depending upon factors such as the particular route of administration. Generally however, compositions will comprise from about 1 to about 98 20 percent by weight of pharmaceutically acceptable carrier, and preferably from 5 to 90 percent by weight of pharmaceutically acceptable carrier.

The size of the dose for therapeutic purposes of a composition 25 of the invention will naturally vary according to the nature and severity of the condition, the age and sex of the animal or patient and the route of administration, according to well known principles of medicine. Generally however, patients are given from 0.5 mg to 75 mg per kg body weight of the immunogenic 30 protein.

Thus in a further aspect, the invention provides the use of a protein as described above in the preparation of a medicament for prophylactic or therapeutic vaccination against *B.* 35 *anthracis*.

As described hereinafter, the applicants isolated and identified loosely adsorbed and likely integral exosporium proteins from spores of *B. anthracis* wild type Ames and *B. anthracis* pX02-plasmid derivative. Proteins were identified from either their amino-terminal sequence and/or Nanospray MS/MS data which provides information on N-terminal, mid-chain and C-terminal sequence with equal probability. These techniques which generate unequivocal identification of sequence were adopted because of the limited resolution of the 1D gels of exosporium material. As only selected bands were examined, and some material did not enter the gel, only a small proportion of the total proteins in the exosporium have been identified so far. All of the proteins identified are encoded chromosomally.

There are no obvious differences in gross exosporium profile between the wild type and cured strains. Therefore the cured strains represent good models for studying the exosporium from spores of *B. anthracis*.

Two proteins, ExsK (SEQ ID NO 7) and ExsF (BxpB) (SEQ ID NO 3), have no homologues of identified function and no homologues in *B. subtilis*, the paradigm sporeformer. However, ExsF, which has been described recently by others (Steichen et al., 2003; Todd et al., 2003) is also present in both *B. cereus* and *B. anthracis*, and is more strongly conserved (97% identity). Thus, although it may be particularly useful as a vaccine, its use in detecting specifically *B. anthracis* is less pronounced.

Three of the exosporium proteins which the applicants isolated were related to outer spore coat proteins of *B. subtilis* spores, CotB (Donovan et al., 1997), CotY (Zhang et al., 1993) and a CotY homologue designated ExsY. CotB is exposed on the spore surface of *B. subtilis* (Donovan et al., 1987; Driks, 1999), 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 techniques. ExsY is required for

exosporium assembly (M. Johnson, S.J. Todd and A. Moir, unpublished). The *cotY* gene forms part of a complex operon in *B. subtilis* which consists of five genes, *cotVWXYZ*. It is hypothesised 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., 1994) There are no ORFs homologous to Cot V, W or X proteins in the *B. anthracis* genome.

The two enzymes, alanine racemase and the inosine preferring nucleoside hydrolase were present on the washed exosporium and as such represent proteins which are tightly associated to this layer. 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 and Hanna, 2002; Titball and Manchee, 1987). These enzymes may be involved in metabolism of germinants and presumably moderate the rate of spore germination.

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 stationary phase of growth (Mignot et al., 2002) and its presence in the exosporium preparations is likely to result from co-purification of the two lattice like structures, exosporium and S-layer, during the exosporium removal and purification procedure. The extent of cross-contamination with EA1 varied in different preparations.

The presence of BclA in unwashed and washed exosporium of *B. anthracis* and *B. cereus* exosporium was suggested by immunoblotting with anti-spore antibodies, but this protein was not amenable to MS/MS sequencing. Difficulties in dissociating proteins e.g. ExsY (SEQ ID NO 5), CotY (SEQ ID NO 4) and ExsF (SEQ ID NO 3) suggest that they are involved in relatively stable multimeric complexes along with the glycoprotein(s) in the exosporium.

Unwashed exosporium contains, in addition to the demonstrated salt and detergent-resistant components, other components, 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.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings, a brief description of which follows.

20

Figure 1 Transmission electron micrographs of *B. anthracis* spores and exosporium

Negatively stained samples of (A) *B. anthracis* UM23C12 spores sonicated for 6 minutes at 12 μ m and (B) Filtered, salt -washed exosporium from sonicated *B. anthracis* wild type Ames spores.

25

Figure 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 Bis-Tris 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.

35

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 obtained by Nanospray-MS/MS, and unequivocal protein assignments made.

Figure 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 weight marker; Lanes 2 and 3; *B. anthracis* UM23Cl2, unwashed and washed respectively; Lanes 4 and 5, *B. anthracis* pX01⁺/pX02⁻ Ames, unwashed and washed respectively; Lanes 6 and 7, *B. anthracis* wild type Ames, unwashed and washed respectively. Selected bands were subjected to DE-MALDI-TOF-MS and a number of peptides identified by Nanospray-MS/MS. The number in the brackets following the proteins identified relates to the number assigned in Table 2.

Figure 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 hour (Lane 3). SigmaMarker broad range molecular weight marker is in Lane 1.

Figure 5 Glycoprotein detection of proteins from unwashed and fully washed exosporium

Proteins separated by SDS PAGE were visualised using the ECL glycoprotein detection kit (Amersham). Invitrogen Western blotting standard (Lane 1); *B. cereus* ATCC 10876 exosporium, unwashed (Lane 2), and washed (Lane 3); *B. anthracis* UM23Cl2 exosporium unwashed (Lane 4), and washed (Lane 5 and 6); *B.*

anthracis wild type Ames exosporium unwashed (Lane 7), and washed (Lane 8 and 9); and transferrin (Lane 10).

Figure 6 Reactivity of antispore monoclonal antibody, F3/1 B11 to the dominant exosporium antigen

Western blot of glycosylated and deglycosylated exosporium from *B. cereus* ATCC 10876 exosporium (Lanes 2 to 3), *B. anthracis* UM23C12 (Lanes 4 and 5) and *B. anthracis* wild type Ames exosporium (Lanes 6 and 7), probed with anti-spore monoclonal antibody F3/1 B11. Lane 1 shows molecular weight marker and lane 8 contains ovalbumin, which was not detected by the MAb.

Bacterial strains, growth conditions and media. The *Bacillus anthracis* strains used were wild type Ames, a pX02 cured Ames derivative (Ames +/- 1) and UM23C12, a double cured derivative. The wild type Ames and pX02 cured Ames derivative were handled in Advisory Committee on Dangerous Pathogens (ACDP) 3 containment, both as vegetative cells and spores, whereas UM23C12 was grown at ACDP 2. The strains were grown routinely on nutrient agar, blood agar or L-agar or in nutrient broth or L-broth at 37°C.

Spore preparation. Spores of *B. anthracis* were prepared on New Sporulation medium (3.0 g l⁻¹ Difco Tryptone Peptone; 6.0 g l⁻¹ Oxoid bacteriological peptone; 3.0 g l⁻¹ Oxoid yeast extract; 1.5 g l⁻¹ Oxoid Lab Lemco; 1 ml 0.1% MnCl₂·4H₂O; 25 g l⁻¹ Difco Bacto agar) at 37°C, until the cultures contained in excess of 95% free spores. Spores were harvested by centrifugation at 10,000 x 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.

Exosporium removal and purification. Spores were defrosted and centrifuged at 10,000 x g for 10 min at 4°C. Prior to sonication, pellets were resuspended to between approximately 5×10^9 and 2.5×10^{10} spores per ml in 50mM Tris-HCl, 0.5mM EDTA buffer (pH7.5).

5 All subsequent manipulations were at 4°C. To fragment and release exosporium material, spores were sonicated (amplitude 12 μ m) using a Sanyo Soniprep, for seven 1 min bursts, each separated by 2 min cooling on ice. Exosporium fragments were separated from spores by pelleting the latter at 9,000 x g for 5

10 min. The spore pellet(s) were washed once in 50mM Tris-HCl, 0.5mM EDTA (pH 7.5) and the exosporium-containing supernatants were pooled, then centrifuged again (10,000 x g, 15 min) to pellet the remaining spores.

15 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 μ m low-protein binding filters (Acrodisc, Gelman Sciences). The filtrate, containing exosporium, was subjected to a sterility check by inoculating 10 volumes of

20 nutrient or L-broth with 10% of the exosporium preparation and incubating at 37°C with shaking (200 rpm) 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

25 growth of any colonies permitted clearance for use at a lower level of containment. Once cleared as sterile, the exosporium-containing filtrate was concentrated using an Amicon ultra-filtration cell fitted with a polyethersulfone membrane (Millipore, NWWL 10,000) or by pelleting in an ultracentrifuge

30 at 184,000 x g for 1 hour 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

35 Nicholson and Setlow (1990). The technique was optimised using *B. anthracis* UM23C12 exosporium and subsequently applied to

exosporium from the ACDP 3 strains of *B. anthracis*. The exosporium was washed with 1M NaCl, followed by 1 or 2 washes in TEP buffer (50mM Tris-HCl pH7.2, 10mM EDTA, 2mM PMSF) containing 0.1% wv⁻¹ 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 x g for 40 min.

Protein determination. Protein concentrations were determined using the BCA protein assay kit (Pierce) according to the Manufacturers 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 (50mM Tris-HCl, pH10, 8M urea and 2% v⁻¹ β -mercaptoethanol) for 15 minutes or 1 hour, 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% wv⁻¹ phosphotungstic acid in phosphate buffer (pH 7.2). After 15 to 30 seconds 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 60KV.

Gel Electrophoresis. Samples were mixed with an equal volume of sample buffer (62.5 mM Tris-HCl, pH 6.8; 10% v⁻¹ glycerol, 2% wv⁻¹ SDS, 5% v⁻¹ β -mercaptoethanol; 0.1% wv⁻¹ bromophenol blue), and boiled for 5 min, then held on ice for 2 to 10 min and centrifuged briefly (30 sec at 13,000 x g) to remove any insoluble material. The proteins were separated by SDS-PAGE in Tris-glycine buffer (24mM Tris, 192mM glycine and 0.1% wv⁻¹ SDS; (Laemmli, 1970)) at 125V using pre-cast gels (Invitrogen, Paisley), or on NuPAGE Bis-Tris pre-cast gels in MES buffer

(Invitrogen). To separate low molecular weight proteins, samples were boiled in Tricine gel sample buffer (900mM Tris-HCl, pH 8.45; 24% vv⁻¹ glycerol; 8% wv⁻¹ SDS; 0.15% wv⁻¹ Coomassie blue G; 0.005% wv⁻¹ Phenol red and 5% vv⁻¹ β -mercaptoethanol) and separated on precast 16% Tris-Tricine gels (Invitrogen) at 125V using the Tricine running buffer (100mM Tris; 100mM Tricine, 0.1% wv⁻¹ SDS). Gels were stained with Colloidal Blue stain (Invitrogen) according to the Manufacturers instructions. For molecular weight determination, SigmaMarker™ molecular weight 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 10mM CAPS transfer buffer (pH 11.0) with 10% vv⁻¹ methanol. The membranes were stained with 0.1% wv⁻¹ Coomassie® 250 in 40% vv⁻¹ methanol/1% vv⁻¹ acetic acid, destained with 50% vv⁻¹ 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 C₁₈ cartridge. The peptide containing fractions were collected and analysed by Delayed Extraction-Matrix Assisted Laser Desorption Ionisation-Time of Flight Mass Spectrometry (DE-MALDI-TOF-MS). Some peptides were subjected to sequencing using Nanospray-MS/MS at M-Scan Limited.

Sequence data. Sequences from protein bands were used to search preliminary sequence data for *B. anthracis* obtained from the Institute for Genomic Research (TIGR) website at <http://www.tigr.org>, using tblastn (Altschul et al., 1997), and to search ORFs identified 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., in press).

Glycoprotein staining. Proteins separated on Tris-glycine SDS-PAGE gels were electrophoretically transferred onto PVDF membranes, stained and visualised using a Glycoprotein ECL detection kit (Amersham Pharmacia Biotech).

Deglycosylation of exosporium. Trifluoromethanesulfonic acid (TFMS)/anisole (9:1 v/v⁻¹; 50µl) was added to 100-200 µg of the lyophilised exosporium and vortexed. The reaction was refrigerated for 2 hours and stopped by the drop wise addition of 1ml of diethylether/pyridine (1:1) prechilled to -20°C. The sample was spun at 16,000 x g for 8 min, and the pellet resuspended in 1 ml 0.1 M NH₄HCO₃. The resuspended exosporium was dialysed against 0.1M NH₄HCO₃ in 3,500 molecular weight cut off dialysis tubing at 4°C with 3 or 4 buffer changes. The dialysed material was spun at 16, 000 x g for 10 min and the pellet resuspended in 50 to 100µl of 50 mM Tris HCl, 0.5 mM EDTA; pH7.5.

Western blotting. Proteins from SDS-PAGE were transferred onto Hybond™-C Extra membrane (Amersham Life Science). The resulting blots were probed with anti-mouse monoclonal antibodies, F3/1 B11 at 1 - 2 µg ml⁻¹ or AF10 at 2 - 10 µg ml⁻¹. Blots were developed using the ECL Western blotting analysis system (Amersham Pharmacia Biotech), diluting the secondary antibody according to the manufacturer's instructions (generally 1:3000 dilution).

30

RESULTS

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. Sonication of spores for 6 or 7 minutes using the Sanyo Soniprep 150

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ultrasonic disintegrator caused partial fragmentation of the exosporium without disruption of the spore (Figure 1A). Some exosporium material was left attached to the spores. Whole spores were removed by low speed centrifugation, the supernatant
5 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 (Figure 1B). Because of the restrictions of working in ACDP 3 containment, exosporium was released using sonication
10 rather than by a French Press procedure.

Effects of salt-washing the exosporium

A large number of protein bands from concentrated wild type Ames exosporium were separated by SDS-PAGE (Figure 2A, lane 2 and
15 Figure 2B, lane 1). The supernatant from the first centrifugation to concentrate the material after filtration (Figure 2A, lane 3) contained many of the same bands. The exosporium preparation was washed successively with salt (1M NaCl) and detergent (0.1% wv^{-1} SDS) to remove loosely adsorbed
20 proteins. The exosporium protein profile after these washing procedures (Figure 2A, lane 4) is 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. Peptide sequences were searched against predicted open reading frames
30 (ORFs) of *B. anthracis* (provided by T. Read, TIGR). An example of a profile used for analysis is in Fig 2B, and Table 1 lists the proteins identified. The S-layer protein EA1 was frequently detected in protein profiles, from both unwashed and washed *B. anthracis* exosporium, as multiple bands of ca. 98, 75 and 66
35 kDa. These exosporium preparations had not been purified on density gradients, and therefore could contain contaminating S-

layer material. Three of the nine proteins identified (alanine racemase, CotY and ExsY) were also identified in washed exosporium. 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 present in *B. anthracis* exosporium.

10 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.

15 **Identification of proteins in the washed exosporium**

Salt and detergent washed exosporium preparations from all three strains examined show a generally reproducible pattern of proteins on SDS-PAGE on a 16% acrylamide, Tris-tricine buffered gel, except for the variable EA1 S-layer proteins (Figure 3, lanes 3, 5 and 7).

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 (>200kDa; Figure 3).

25 Several protein components were identified - their amino acid sequences, predicted from the *B. anthracis* genome sequence, are shown in Table 2.

The strong band with apparent molecular weight of 43 kDa present in both unwashed and washed exosporium was identified from both MS sequencing (Table 2) and direct N-terminal sequencing (Table 1), as an alanine racemase homologue (50% amino acid identity to the *B. subtilis dal* gene product). A second alanine racemase homologue encoded in the unfinished *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 (Table 2) contains a homologue of inosine-
uridine preferring nucleoside hydrolases (30% amino acid
identity with the paradigm protozoal enzyme of Gopaul et al.,
5 (1996)).

The band at 17 kDa contained sequences that correspond to a
protein, identifiably encoded by *B. anthracis*, and very recently
described and designated BxpB by Steichen et al (2003). The
10 predicted molecular weight of 17 kDa corresponds to that
observed experimentally. The equivalent protein in *B. cereus*
exosporium has been designated ExsF (Todd et al., 2003). A
related paralogue is also encoded in the *B. anthracis* genome
sequence, sequence PVIND is the only sequence obtained present
15 in both genes.

The band at 14 kDa contains sequences derived from at least two
separate proteins. One is a homologue of (30% identity to) *B.*
subtilis spore coat protein CotB (Table 2), of predicted size
20 19.4 kDa. The sequence data do not correspond to the sequence
of a second, rather shorter CotB homologue, encoded in the *B.*
anthracis genome immediately downstream of the first.
The other sequences from the 14kDa band, FEAFAF and SANLT, are
present in the sequences of both ExsY and CotY. These are
25 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).

30 The band at 10 kDa contains peptides from a predicted protein,
which we have named ExsK. This protein has a homologue in the
B. cereus ATCC 14579 genome sequence with 64% identity. Direct
comparison of ExsK with *B. subtilis* proteins by FASTA gives the
highest, though not significant score ($e = 0.12$) as the *B.*
35 *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, suggesting
5 that either or both ExsY and CotY sequences are present. The
band at ca. 6.5 kDa 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 to 17 kDa.
10

Peptide sequences were obtained from the second largest band
(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 weight
15 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* UM23C12 were incubated with alkaline
20 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 (Figure 4) were subjected to MS/MS and N-
terminal sequencing. The diffuse multiple band (>250kDa) in the
25 15 min extract contained peptides from ExsY (SVDDDS) and ExsF
(MFSSD) and yielded the N-terminal sequence for glycoprotein
BclA (AFDPNLVGPT). Specific staining did confirm the presence
of glycoprotein in this region of the gel (Figure 5). On more
extended incubation, a smaller band at 205 kDa increased in
30 intensity; peptides PVIND and PVELI from ExsF were both
identified in this band.

Glycoprotein(s) in the exosporium

SDS-PAGE separated *B. anthracis* exosporium proteins were tested
35 for glycoprotein using an ECL glycoprotein detection system
(Amersham Pharmacia Biotech) and compared to those of *B. cereus*

(Figure 5). A large diffuse band extending from the insoluble fraction at the top of the gel, to a molecular weight of approximately 140 kDa was stained in the *B. anthracis* exosporium preparations. *B. cereus* has a second diffuse band at 70 kDa.

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Monoclonal antibodies F3/1 B11, raised against bacterial spores of *B. anthracis* wild type Ames (C. Redmond, unpublished) or AF10, raised against irradiated *B. anthracis* Sterne spores by Dr J. Kearney, were used as probes in Western blots of SDS-PAGE separated exosporium extracts. Both bound to the insoluble fraction at the top of the gel and to a band at >200kDa (Figure 6).

Exosporium preparations were chemically deglycosylated using trifluoromethanesulfonic acid (TFMS). Following deglycosylation, antibodies AF10 and F3/1 B11 detect protein bands at apparent molecular weights of 42 kDa in *B. cereus* and approximately 66 kDa in both *B. anthracis* Ames and UM23C12 (Figure 6). The deglycosylated exosporium samples were analysed for the presence of glycoprotein and bands with sizes corresponding to those detected by the antibodies were stained (data not shown). Therefore complete deglycosylation of the exosporium glycoproteins was not achieved. A BclA specific antibody (described in Sylvestre *et al.*, 2002) also detected proteins in identical positions to those detected by AF10 and F3/1 B11. Therefore AF10 and F3/1 B11 must also be detecting BclA. The *B. anthracis* bands detected are smaller than reported for the Sterne strain in Sylvestre *et al* (2002); the length of BclA has been shown to vary in different *B. anthracis* isolates (Steichen *et al.*, 2003; Sylvestre *et al.*, 2002; 2003). The size of the protein detected in deglycosylated anthrax exosporium by the antibodies was larger than the molecular weight of BclA (35kDa) predicted from the genome sequence of the Ames strain.

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References

- Altschul, S. F., Madden, T. L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J.** (1997). *Nucl. Acids Res* **25**, 3389-3402.
- 5 **Beaman, T.C., Pankratz, H.S., & Gerhardt, P.** (1971). Paracrystalline sheets reaggregated from solubilized exosporium of *Bacillus cereus*. *JBacteriol*, **107**, 320-324.
- 10 **Charlton, S., Moir, A.J.G., Baillie, L. & Moir, A.** (1999). Characterisation of the exosporium of *Bacillus cereus*. *J ApplMicrobiol* **87**, 241-245.
- DesRosier, J.P., & Lara, J.C.** (1984). Synthesis of the
15 exosporium during sporulation of *Bacillus cereus*. *J GenMicrobiol* **130**, 935-940.
- Dixon, T.C., Fadl, A. A., Koehler, T. M., Swanson, J. A., & Hanna, P.** (2000). Early *Bacillus anthracis* macrophage
20 interactions: intracellular survival and escape. *Cell. Microbiol.* **2**, 453-463.
- Donovan, W., Zheng, L., Sandman, K., & Losick, R.** (1987). Genes encoding spore coat polypeptides from *Bacillus subtilis*. *J*
25 *MolBiol* **196**, 1-10.
- Driks, A.** (1999) *Bacillus subtilis* spore coat. *Microbiol Mol Biol Rev*, **63**, 1-20.
- 30 **Edge, A. S. B., Faltynek, C. R., Hof, L., Reichert, Jr L. E., & Weber, P.** (1981). Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *AnalBiochem* **118**, 131-137.
- Garcia-Patrone, M., & Tandecarz, J.S.** (1995). A glycoprotein
35 multimer from *Bacillus thuringiensis* sporangia: Dissociation

into subunits and sugar composition. *Molec Cell Biochem* **145**, 29-37.

Gerhardt, P., & Ribí, E. (1964) Ultrastructure of the exosporium
5 enveloping spores of *Bacillus cereus*. *J Bact* **88**, 1774-1789.

Gopaul, D. N., Meyer, S. L., Degano, M., Sacchettini, J. C. & Schramm, V. L. (1996). *Biochemistry* **35**, 5963-5970.

10 **Guidi-Rontani C., Levy, M., Ohayon, H., & Mock M.** (2001). Fate of germinated *Bacillus anthracis* spores in primary murine macrophages. *Mol Microbiol* **42**, 931-938.

Hachisuka, Y., Kojima, K., and Sato, T. (1966). Fine filaments
15 on the outside of the exosporium of *Bacillus anthracis* spores. *J Bacteriol* **91**, 2382-2384.

Hakimuddin, T., Sojar H.T., & Bahl O.P. (1987a). A chemical method for the deglycosylation of proteins. *Arch Biochem Biophys*
20 **259**, 52-57. Di you really need three refs for glycoprotein deglycosylation? Are all still useful?

Hakimuddin, T., Sojar H.T., & Bahl O.P. (1987b). Chemical
deglycosylation of glycoproteins. *Meth Enzymol* **138**, 341-50.

25

Hills, G. M. (1949). Chemical factors in the germination of spore bearing aerobes. The effects of amino acids on the germination of *Bacillus anthracis*, with some observations on the relation of optical form to biological activity. *Biochem. J* **45**,
30 363-370.

Ireland, J.A.W., & Hanna, P.C. (2002). Macrophage-enhanced germination of *Bacillus anthracis* endospores requires *gerS*. *Infect Immun* **70**, 5870-5872.

35

- Isticato, R., Cangiano, G., Tran, H.T., Ciabattini, A., Medaglini, D., Oggioni, M.R., De Felice, M., Pozzi, G., & Ricca, E. (2001). Surface display of recombinant proteins on *Bacillus subtilis* spores. *J Bacteriol* **183**, 6294-6301.
- 5 **Kramer, M.J., & Roth, I.L.** (1968). Ultrastructural differences in the exosporium of the Sterne and Vollum strains of *Bacillus anthracis*. *Can.J Microbiol* **14**, 1297-1299.
- 10 **Lai, E.M., Phadke, N.D., Kachman, M.T., Giorno, R., Vazquez, S., Vazquez, J.A., Maddock, J.R., & Driks A.** (2003). Proteomic analysis of the spore coats of *Bacillus subtilis* and *Bacillus anthracis*. *J Bacteriol* **185**, 1443-54.
- 15 **Laemmli, U.K.** (1970). Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-689.
- Matz, L.L., Beaman, C., & Gerhardt, P.** (1970). Chemical
20 composition of exosporium from spores of *Bacillus cereus*. *J Bacteriol* **101**, 196-201.
- Mignot, T., Mesnage, S., Couture-Tosi, E., Mock, M., & Fouet, A.**
(2002). Developmental switch of S-layer protein synthesis in
25 *Bacillus anthracis*. *Mol Microbiol* **43**, 1615-1627.
- Moberly, B.J., Shafa, F., & Gerhardt, P.** (1966). Structural details of anthrax spores during stages of transformation to vegetative cells. *J Bacteriol* **92**, 220-228.
- 30 **Nicholson, W.L., & Setlow, P.** (1990). Sporulation, germination and outgrowth. In *Molecular and Biological Methods for Bacillus*, eds Harwood, C.R., and Cutting, S.M. (John Wiley and Sons, Chichester). pp.391-450.

- Read, T.D., Peterson, S.N., Tourasse, N., Baillie, L.W., Paulsen, I.T., Nelson, K.E., Tettelin, H., Fouts, D.E., Eisen, J.A., Gill, S.R., Holtzapple, E.K., OKstad, O.A., Helgason, E., Rilstone, J., Wu, M., Kolonay, J.F., Beanan, M.J., Dodson, R.J.,
5 Brinkac, L.M., Gwinn, M., DeBoy, R.T., Madpu, R., Daugherty, S.C., Durkin, A.S., Haft, D.H., Nelson, W.C., Peterson, J.D., Pop, M., Khouri, H.M., Radune, D., Benton, J.L., Mahamoud, Y., Jiang, L., Hance, I.R., Weidman, J.F., Berry, K.J., Plaut, R.D., Wolf, A.M., Watkins, K.L., Nierman, W.C., Hazen, A., Cline, R.,
10 Redmond, C., Thwaite, J.E., White, O., Salzberg, S.L., Thomason, B., Friedlander, A.M., Koehler, T.M., Hanna, P.C., Kolsto, A.B., & Fraser, C.M. (2003). The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* **423**, 81-86.
- 15 Roth, I.L., & Williams, R.P. (1963). Comparison of the ultrastructure of virulent and avirulent spores of *Bacillus anthracis*. *Texas Report of Biological Medicine* **21**: 394-399.
- 20 Steichen., C., Chen, P., Kearney, J.F., & Turnbough, C.L. Jr. (2003). Identification of the immunodominant protein and other proteins of the *Bacillus anthracis* exosporium. *J Bacteriol* **185**, 1903-1910.
- 25 Sylvestre, P., Couture-Tosi, E., & Mock, M. (2002). A collagen-like surface glycoprotein is a structural component of the *Bacillus anthracis* exosporium. *Mol Microbiol* **45**, 169-178.
- Sylvestre, P., Couture-Tosi, E., & Mock, M. (2003). Polymorphism
30 in the collagen-like region of the *Bacillus anthracis* BclA protein leads to variation in exosporium filament length. *J Bacteriol* **185**, 1555-1563.
- 35 Titball, R. W., & Manchee. R.J. (1987). Factors affecting the germination of spores of *Bacillus anthracis*. *J Appl Microbiol* **62**, 269-273.

- Todd, S.J., Moir, A.J.G., Johnson, M.J., and Moir, A.** (2003) Genes of *Bacillus cereus* and *Bacillus anthracis* encoding proteins of the exosporium. *J Bacteriol* **185**, 3373-3378.
- 5 **Zhang, J., Fitz-James, P.C & Aronson, A. I.** (1993). Cloning and characterization of a cluster of genes encoding polypeptides present in the insoluble fraction of the spore coat of *Bacillus subtilis*. *J Mol Biol* **175**, 3757-3766.

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

Gene Identification (Protein)	Apparent Mr	Predicted Mr	Predicted pI	N-terminal sequence	Peptide sequences
BA0252 Alanine racemase	48 kDa	43.7 kDa	5.51	MEEAPFYRDT ^a	
BA0267 GroEL	61 kDa	57.4 kDa	4.79		ELEDAFENM ^b VGNDGVITIEESK ^b
BA1629 Cold shock protein (CspB-1)	7.3 kDa	7.2 kDa	4.60	MQNGKVKQFNSEK ^b	
BA1238 CotY	17.8 kDa	16.8 kDa	4.71	SLNXNEDHHHHDXDFNXV ^a	
BA1234 ExsY	16.1 kDa (N-terminal sequencing) >250 kDa (MS)	16.1 kDa	4.95	SXNENKHHGSXHNVVDEVKF ^a SVDDDDSCAVLR ^b	SVDDDSACAVLR ^b PFEAFA ^b
BA2332 ExsL	14.4 kDa	14.4 kDa	5.24	MFFAKLRGRNEVPVETDARG ^a	
BA4181 Dihydrolipoamide dehydrogenase (pdhD)	50 kDa	49.5 kDa	5.26		AYFVDAN ^b
BA0108 Translation elongation factor Tu	43 kDa	42.9 kDa	4.93		DQIDAA ^b
BA4387 Leucine dehydrogenase	40 kDa	39.9 kDa	5.13		FGTDNLEGG ^b

Sequences identified from:

^a Ames +/- 1 exosporium^b Wild type Ames exosporium

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

Gene Identification (Protein)	Apparent Mr (kDa)	Predicted Mr (kDa)	Predicted pI	Peptide sequences
BA0252 Alanine racemase (dal)	43	43.6	5.51	HTANS (Band 4); VLVNGK (Band 4) MEEAPFYRDTWVEVDLDAIYNNVTHIKEFIPSDVEIFAVVKGNAVGHYDVPVAKIALEAG ATRLAVAFLEALVLRAGITAPILVLGPPPRDINVAEENDVALTVFQKEWVDEAIKLW DGSSTMKYHINFDSGMGRIGIRERKELKGFLKSLEGAPFLELGVYTHFATADEVETSYF DKQYNTFLEQLSWLKEFGVDPKFVHTANSAAATLRFQGITFNNAVRIGIAMYGGLSPSVEIRP FLPFKLEPALSLHTKVAHIKQVIKGDGISYNNVTYRTKTEEWIATVAIGYADGWLRRLQGF EVLVNGKRVPIVGRVTMDQFMHLPCEVPLGTVKTLIGRQGEYISATEVAEYSGTINYE IITTISFRVPRIFIRNGKVVEVINYLNDI (SEQ ID NO 1)
BA2888 Inosine- preferring nucleoside hydrolase	33	36.3	5.05	VTENVYFLER (Band 5); ISFINDD (Band 5) MSIVNKKIIFFGDFGIDDAVALIYANKTCKLDILGIVAEYGNVSREIVTENVYFLERYYA TEVKIIEGAARPMATAEEPLFFPEIHGEHGLGPIIPDLRICKRENFCELIKLIEPCPEDI IIVATGRLTTLATLFLLYPNVMDKVCSSYIMGGAFLLFPGNVTPVSEANFYGDPIAANIVM KYAKNAHIYPLNVLTQEALITPEMVDIINKEGTGQAKLIKPMIDFYENFYKKEYPGIAGS PIHDLPLPFI ~~SFINDD~~ IFEYKKS ~~AVWISTTNDVTRGQSVADFRKIAEPTRFDDRPVQKIAV~~ GFNYPAFKEEFMRITILKPDCEP (SEQ ID NO 2)
BA1237 hypothetical protein (ExsF)	17	17.3	4.43	MFSSD (Band 11 & 12); TISLDNS (Band 6); PVIND (Band 13); PVELI (Band 6 & 13). MFSSDCEFTKIDCEAKPASTLPAFGFAFNASAPQFASLFTPLLLPSVSPNPNTITVPVIND TVSVGDGIRILRAGIYQISYTLT ~~TISLDNSPVAP~~ EAGRFFLSLGTTPANILPGSGTAVRSNV IGTGEVDVSSGVILINLNP ~~GD~~ LIRIVPVELIGTVDIRAAALTVAQIS (SEQ ID NO 3)

30

Gene Identification (Protein)	Apparent Mr (kDa)	Predicted Mr (kDa)	Predicted pI	Peptide sequences
BA1238 spore coat protein Z (cotZ) CotY	14 and 6.5 kDa	16.8	4.71	FEAFAP (Band 7, 8 & 11); SANLT (Band 9); SIDDDD (Band 9) MSCNCNEDHHHDCDFNCVSNVVRFIHELQECATTCGSGCEVPFLGAHNSASVANTRPF ILYTKAGAPFEAFAPSANLTSCRSPIFRVESIDDDCAVLRVLSVVLGDTSPVPPTDDPI CTFLAVPNARLISTNTCLTVDLSCFCAIQCLRDVTI (SEQ ID NO 4)
BA1234 spore coat protein Z (cotZ) ExsY	14 and 10 kDa	16.1	4.95	FEAFAP (Band 7, 8 & 11); SVDDDS (Band 12) MSCNENKHGSSHCVVVDVVKFINELQDCSTTCGSGCEIPFLGAHNTASVANTRPFILYT KAGAPFEAFAPSANLTSCRSPIFRVESVDDDDSCAVLRVLSVVLGDTSPVPPTDDPCTFL AVPNARLVSTSTCITVDLSCFCAIQCLRDVTI (SEQ ID NO 5)
BA0355 CotB Homologue	14	19.4	5.31	ISVCPD (Band 7) MSLFHCDFLKDLIGSFVRVNRGGPESQRTIISVCPDYFVLQNEKGELYYYQLSHLKSIT KSAKECGSSDCEWEDCAEDFEALLESFKYCWVKINRGGPEKVEGILQDVSCDFVTLIV KEEIIILIAIKHIKSVNYNALACGESEDDSSSKESNNNSGRARAQRQSSRGR (SEQ ID NO 6)
BA2554 hypothetical protein ExsK	10	12.0	7.96	NYAGTVS (Band 8) MGSRYNSRKKCKSCQCKQDDCWDFECKKEHEEQNKACDCCVQGI RDELRLKLVNR SVRITTGSNNYAGTVSSVTCDVVKLANAGVVTVIISVCKIEAIEALLT (SEQ ID NO 7)

ORF numbers from TIGR genome sequence

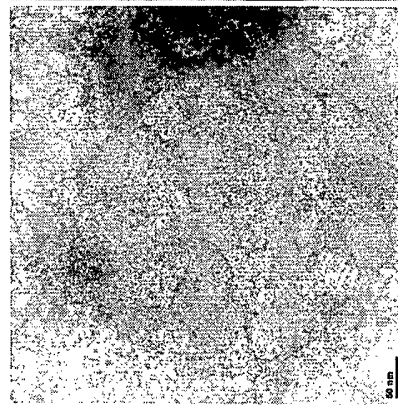
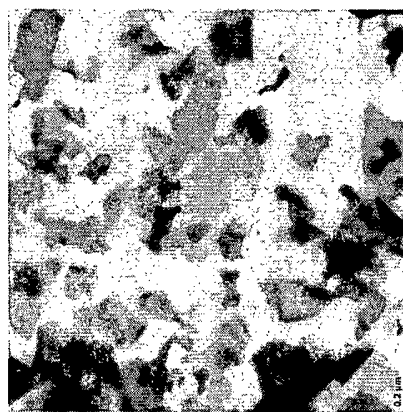
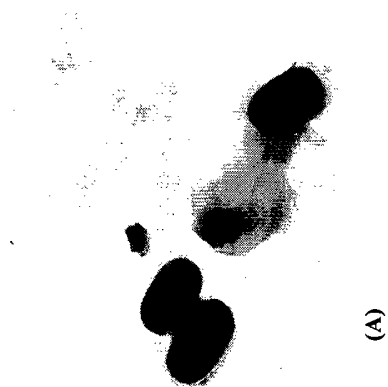
Claims

1. A a protein obtainable from an outer layer of *B. anthracis* in a pre-vegetative state, which protein is either (a) retained in said layer after washing with a salt solution, followed by washing with detergent, and/or (b) retained in a supernatant of said layer which supernatant is obtained after having first removed the exosporium from said *B. anthracis* in a pre-vegetative state; wherein said protein has one or more regions specific to *B. anthracis*, or a variant of said protein, or a fragment of any of these, for use in producing an immunogenic response to *B. anthracis*.
2. A protein according to claim 1 for use in producing an antibody, useful in the detection of *B. anthracis*.
3. A protein according to claim 1 for use in producing an immune response in a host animal, which response is protective against infection by *B. anthracis*.
4. A protein according to any one of the preceding claims which is retained in said outer layer after washing with salt solution, followed by washing with detergent.
5. A protein according to claim 4 wherein the outer layer is the exosporium.
6. A protein according to claim 5 which is a protein of SEQ ID NO 1, 2, 3, 4, 5, 6 or 7 as shown in Table 2, or a variant or fragment thereof.
7. A protein according to claim 6 which is a protein of SEQ ID NO 6 or 7, or a variant or fragment thereof.
8. A protein according to claim 1 which is retained in a supernatant of said layer which supernatant is obtained

after having first removed the exosporium from said *B. anthracis* in a pre-vegetative state.

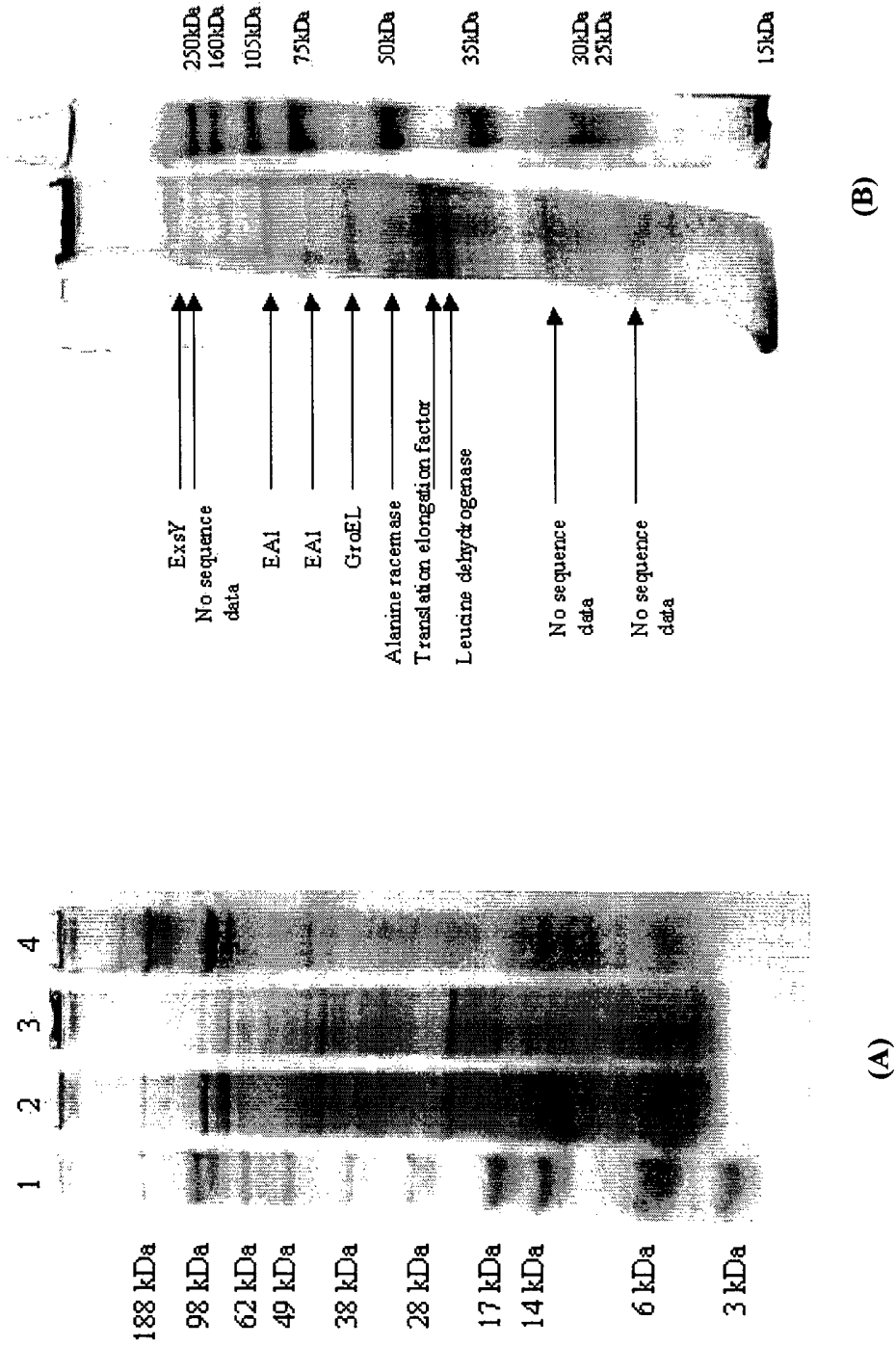
9. A protein according to Claim 8 wherein the protein has less than 80% sequence identity to any protein obtainable in a similar manner from another *Bacillus* species.
10. A protein according to claim 9 which is protein ExsL as defined in Table 1.
11. A protein comprising SEQ ID NO 7 or a variant having greater than 64% identity thereto, or immunogenic fragment thereof.
12. A protein according to claim 11 which comprises SEQ ID NO 7.
13. A binding moiety comprising an antibody or a binding fragment thereof, which specifically recognises a protein according to any one of the preceding claims.
14. A method for detecting the presence of *B. anthracis* which method comprises contacting a sample suspected of containing *B. anthracis* cells with a binding moiety according to claim 13 and detecting binding therebetween.
15. A pharmaceutical composition comprising a protein according to any one of claims 1 to 13 in combination with a pharmaceutically acceptable carrier.
16. The use of a protein according to any one of claims 1 to 13 in the preparation of a medicament for prophylactic or therapeutic vaccination against *B. anthracis*.
17. An isolated nucleic acid which encodes a protein according to claim 11 or claim 12.

Figure 1



(B)

Figure 2



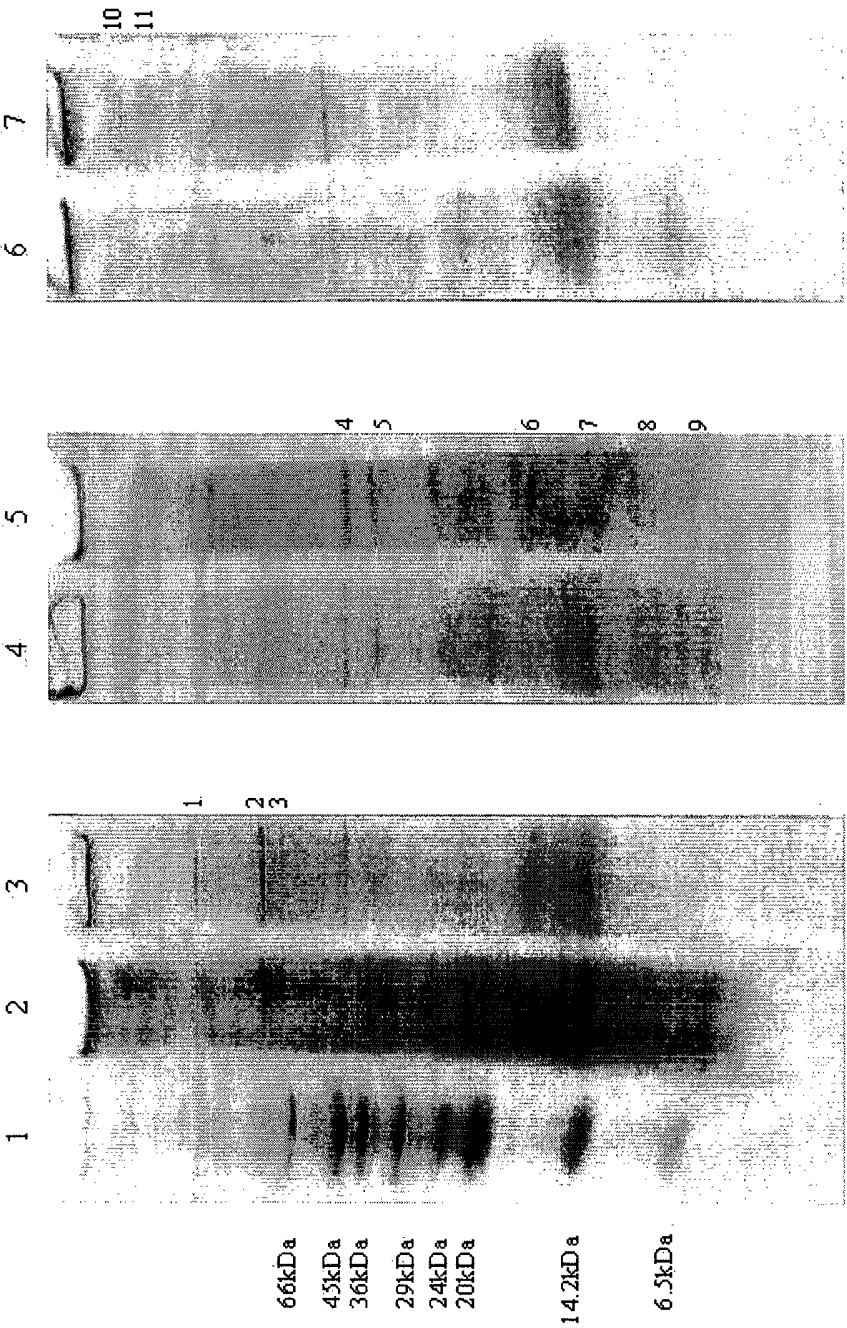


Figure 3



Figure 4

Figure 5

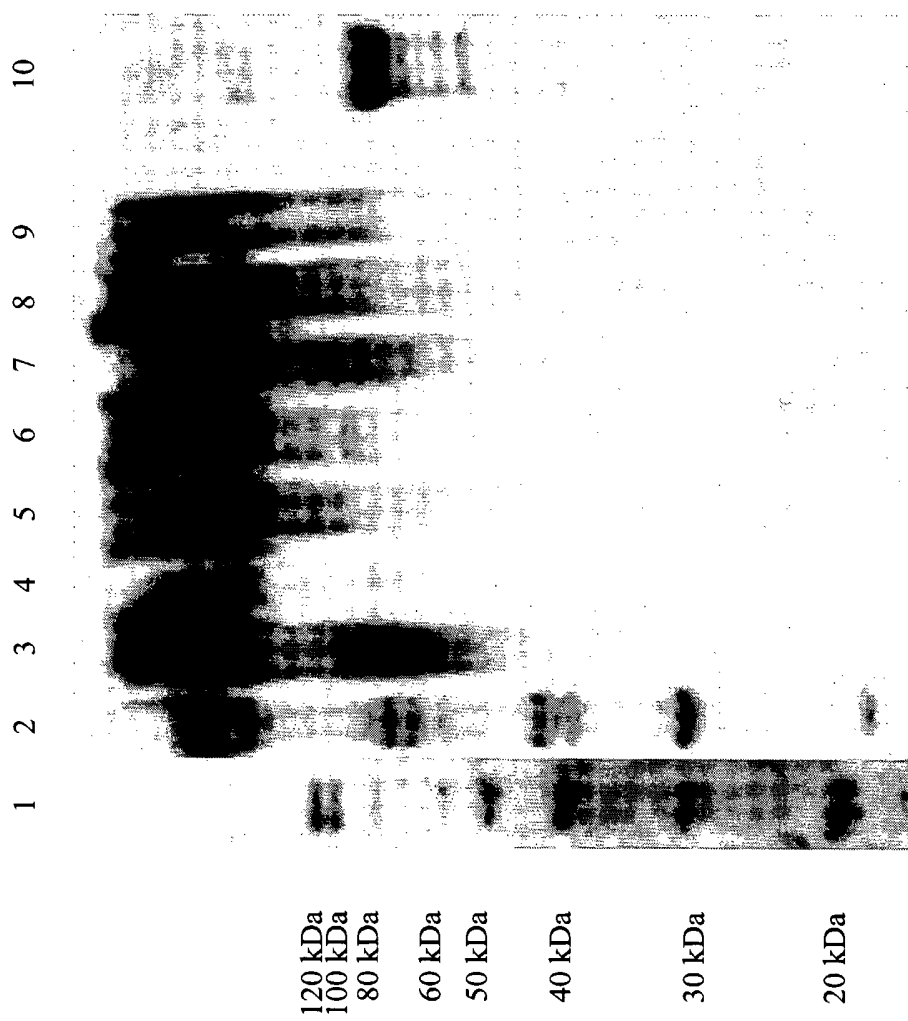


Figure 6

