Abstract: The invention provides a non-pathogenic spore comprising an antigenic fragment of anthrax protective antigen for use as an anthrax vaccine particularly by nasal and/or oral administration.

ELISA titers vs time for Nasal Immunisation with spore coat expression constructs
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ANTHRAX VACCINE IN THE FORM OF A SPORE

The present invention relates to a new vaccine for anthrax, a method of inducing an immune response against anthrax and a method of preparing an anthrax vaccine.

Anthrax is an acute infectious disease caused by the spore forming bacterium *Bacillus anthracis*. The disease takes three forms in humans; cutaneous, gastrointestinal and inhalation (pulmonary anthrax) with the gastrointestinal and inhalation forms being the most deadly with mortality rates of up to 90%. The anthrax bacterium is found globally, present in the soil as extremely resistant spores and most commonly causes disease in hoofed animals. There are an estimated 20,000 to 100,000 human cases of anthrax per year, most of which occur in the developing world. However, in the US, the annual incidence had declined to less than 1 case per year. The recent use of anthrax as a bioterrorism agent in the US in October to December 2001 clearly demonstrated the potential of this bacterium as a biowarfare agent with 12 cases of clinically confirmed anthrax documented. These included 6 cases of inhalation anthrax (3 fatal) and 6 cases of cutaneous anthrax. Anthrax is currently recognised alongside smallpox as the most likely biological warfare agent by Western governments.

The current veterinary vaccine is a spore preparation of a *B. anthracis* strain (the Sterne strain) lacking the pXO2 plasmid. This strain produces anthrax toxin but is unable to synthesise the capsule required for survival in host cells. While effective this veterinary vaccine has a low level of virulence (since it still produces the anthrax toxin) and can produce necrosis at the site of injection (Hambleton & Turnbull, 1990). In contrast, the human vaccine is a non-cellular vaccine (AVA) produced in the UK and USA from a toxigenic, non-capsulated, Sterne strain by
adsorbing culture supernatant to aluminium hydroxide. The key component of this vaccine is the 82.7 kDa protective antigen (PA). While efficacy has been established in various animal models, vaccination requires multiple doses and boosters (six injections over 18 months). Adverse side effects have been reported (erythema, induration) and in some animals the vaccine does not provide protection against all virulent B. anthracis strains (Hambleton & Turnbull, 1990). Finally the AVA vaccine is expensive to produce. Recent work has shown that anthrax vaccination is greatly enhanced if B. anthracis spores of the Sterne strain are included in the vaccine together with PA and that the spores themselves play a critical role in stimulating protective immunity (Brossier et al., 2002).

Protective antigen (PA; mwt. 82.7 kDa) is the binding/translocating component of both toxins (LeTx and EdTx) produced by B. anthracis and encoded by the pag gene. Administered alone, PA is not toxic but must associate with lethal factor (LF) to produce LeTx and edema factor (EF) to produce EdTx. PA is secreted from the B. anthracis cell and is involved in receptor binding to host cells and then for translocation of PA-EF and PA-LF to the cell cytoplasm. PA is protective, that is, immunisation of an animal model with this protein can alone provide protection to challenge with B. anthracis.

Current vaccine strategies require provision of protective antigen to the vaccinee using either purified PA or purified PA plus attenuated/inactive spores of B. anthracis.

Support for new vaccine development is provided primarily by defence and antiterrorism measures within national programmes in the UK e.g. the Ministry of Defence and the Department of Defence, often supported
by national health research agencies such as the Medical Research Council and the National Institute of Health.

Many pathogens are able to initiate disease through infection of the mucosal surfaces. The infectious agent first makes contact with, and then colonises (or transverses), the mucosal surface to infect the host (e.g., HIV, TB). Traditional vaccination strategies relying on parenteral immunisation are unable to prevent the initial interaction of pathogen and host at the mucosal surface but rather resolve the resulting infection (Walker, 1994). Oral (or intranasal) immunisation should induce secretory IgA antibodies directed against the specific pathogen as well as CTL responses from CD8+ MHC restricted cytotoxic T-lymphocytes located in the mucosal epithelium and draining lymph nodes (LN) providing an effective means for preventing infection. One problem with oral immunisation, though, is that orally administered antigens frequently lose their immunogenicity. These limitations can be countered using attenuated and live bacteria (e.g. *Salmonella* spp.) that act as carriers of heterologous antigens. One of the most pervasive limitations to the use of bacteria as vaccine vehicles though is their lack of heat stability. A vaccine carrier that can induce mucosal immunity, that can be used orally or nasally and is heat-stable is obviously attractive for development of the next phase of improved vaccines. Further improvements in vaccines against anthrax have been sought.

According to the invention there is provided a non-pathogenic spore comprising an antigenic fragment of anthrax protective antigen.

According to the invention there is also provided a pharmaceutical composition comprising a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen in association with a pharmaceutically acceptable carrier and/or excipient.
According to the invention there is further provided a spore according to
the invention for use as an anthrax vaccine.

According to the invention there is also provided a pharmaceutical
composition according to the invention for use as an anthrax vaccine.

According to the invention there is further provided use of a spore
according to the invention or of a pharmaceutical composition according
to the invention in the manufacture of a medicament for use as an anthrax
vaccine.

According to the invention there is further provided use of a non-
pathogenic spore comprising at least an antigenic fragment of anthrax
protective antigen in the manufacture of a vaccine for anthrax for non-
parenteral administration, preferably nasal and/or oral administration.

According to the invention there is further provided a method of inducing
immunity to anthrax in a mammal susceptible to anthrax infection which
method comprises non-parenteral administration, preferably nasal and/or
oral administration of an effective amount of a vaccine comprising a non-
pathogenic spore comprising at least an antigenic fragment of anthrax
protective antigen

According to the invention there is further provided a method of inducing
immunity to anthrax in a mammal susceptible to anthrax infection which
method comprises administration of an effective amount of a vaccine
comprising a spore according to the invention or a composition according
to the invention to the mammal.
Advantages of the invention include that it is not necessary to use a live attenuated *B. anthracis* strain. This is advantageous for ethical reasons where it is not acceptable, long term, to be using an attenuated pathogen and enables the vaccine to be used with vaccines having a compromised immune response. A further advantage is that *B. subtilis* which is a preferred source of a spore according to the invention is relatively easy and cheap to produce.

Another advantage is that the invention provides a spore-specific immune response to anthrax which is important because this is one way in which anthrax is transmitted. Even though the spores used in the invention are non-pathogenic and therefore not of *B. anthracis*, they will still generate a spore-specific response, enhancing the efficacy of protection. Anti-PA antibodies have been shown to stimulate phagocytosis of *B. anthracis* spores while inhibiting their germination within the phagolysosome. This appears to be an efficient method for dealing with pathogenic spores. How spore germination is inhibited is unclear but presumably binding of anti-PA antibodies to the spore prevents entry of germinants. It has been proposed that the phagosome/phagolysosome somehow provides a germination signal (Guidi-Rontani *et al.*, 1999).

A further advantage to the invention is that it has been found that the spore according to the invention germinates within a phagocyte. It is known that *B. anthracis* spores germinate in macrophages which is critical for pathogenesis and contributes to cellular responses (Th1 dependant) as well as enhancing humoral immune responses (probably TH2 dependant). Thus it is believed that this ability of spores to persist in macrophages is a common phenomenon to *Bacillus* spores and serves to elicit cellular responses. Accordingly the use of a non-pathogenic spore for antigen delivery mimics the fate of *B. anthracis* spores and enhances
vaccine potency. Furthermore it has surprisingly been found that a spore according to the invention can be used to generate mucosal immunity.

The non-pathogenic spore is preferably from a *Bacillus* species spore. More preferably the spore is from one or more of the following organisms: *Bacillus alvei; Bacillus badius; Bacillus brevis; Bacillus cereus; Bacillus coagulans; Bacillus fastidiosus; Bacillus licheniformis; Bacillus mycoides; Bacillus pasteurii; Bacillus sphaericus; Bacillus aneurinolyticus; Bacillus carotarum; Bacillus flexus; Bacillus freudenreichi; Bacillus macroides; Bacillus similibadius; Bacillus thiaminolyticus; Bacillus subtilis; Bacillus pumilus; Bacillus vallismortis; Bacillus bengalicus; Bacillus flexus; and/or Bacillus licheniformis. Most preferably, the spore is from *Bacillus subtilis*.

The spore is non-pathogenic. This generally means that neither the spore nor a bacterium into which the spore may germinate is harmful to the host to which the spore is to be administered.

*B. subtilis* is a ubiquitous, Gram positive, non-pathogenic organism, normally found in the soil. The spore is a dormant life form that can resist extreme environmental conditions (Nicholson et al., 2000) and has a number of attributes making them particularly suitable for the development of a generic vaccine system, these are:-

- Dormant with suitable storage and desiccation properties,
- Suitable for non-parenteral delivery, particularly by the oral and nasal route,
- No evidence for compromised immune status in man,
- Can be deactivated, e.g. with gamma radiation, if necessary,
- Easily modified genetically,
- Can be produced in large quantities; safely, efficiently and cost effectively,
Robust, can survive indefinitely at temperatures up to 90°C,
Suitable for field use, particularly in developing countries,
Resistant to UV irradiation and desiccation,
Currently used in Europe as a probiotic for human use.

The spore is optionally either a germinating spore or a non-germinating spore. Where the spore is non-germinating, it has preferably been treated to prevent germination. Germination can be prevented by using gamma irradiation or by using a germination-deficient mutant spore (such as that disclosed in Duc et al., 2003a).

The spore is preferably a germinating spore. This is because it has been found that an improved immunogenic response can be obtained with a vaccine using such a spore.

Where the non-pathogenic spore used in the invention comprises at least an antigenic fragment of anthrax protective antigen, it is to be understood that it comprises either anthrax protective antigen or a fragment thereof. The antigenic fragment of anthrax protective antigen used in the invention is generally a fragment of anthrax protective antigen which is sufficient to stimulate a suitable immunogenic response.

The spore may optionally comprise at least an antigenic fragment of anthrax protective antigen in the form of a protein attached to the spore (preferably the at least an antigenic fragment is attached to the proteinaceous coat of the spore) and/or in the form of DNA which encodes at least an antigenic fragment of anthrax protective antigen which DNA is adapted to be expressed when the spore germinates.

Where the at least an antigenic fragment of anthrax protective antigen is provided in the form of DNA, the DNA is preferably under the control of
a vegetative cell promoter so that the DNA is only expressed when the spore germinates and/or begins to outgrow. A suitable promoter is for example *rnrO*.

In the present invention, the at least an antigenic fragment of anthrax protective antigen is preferably either PA83 (full length) (SEQ ID No. 16) or a fragment thereof. A fragment of PA83 (full length) is preferably one or more of PA83 (SEQ ID No. 6), PA63 (SEQ ID No. 7), Domain 1 of PA (SEQ ID No. 1), Domain 2 of PA (SEQ ID No. 2), Domain 3 of PA (SEQ ID No. 3), Domain 4 of PA (SEQ ID No. 4) and Domain D1b23 of PA (SEQ ID No. 10).

There are two optimal routes for expression on a spore which can be used which are fusion of the DNA encoding the at least an antigenic fragment of anthrax protective protein and/or a truncated form to CotB or CotC with a suitable promoter for CotB or CotC. In each case genetic engineering is used to splice the *cotB* or *cotC* genes to the *B. anthracis* sequences encoding PA83 (full length) or a fragment thereof.

To express the antigenic fragment of anthrax protective antigen in a germinating spore, the same sequences are fused to an expression cassette.

The sequence of a promoter used in the invention is SEQ ID No. 5. It allows convenient insertion of any ORF (open reading frame) downstream of strong transcriptional and translational signals. The preferred transcriptional signals are comprised of the −35 and −10 promoter sequences of the *rrnO* gene of *B. subtilis*.

The translational signals are provided by placing the start codon and ribosome binding site of the *sspA* gene of *B. subtilis*. *sspA* encodes a
small acid soluble protein that is expressed during sporulation. Expression using this RBS is high.

The sequence of the chimeric promoter Prrn0-RBS(sspA) is shown in Figure 15 and arrangements shown schematically in Figs 2 and 3. Immediately downstream of the ATG start codon is a multiple cloning site (MCS) carrying numerous restriction endonuclease sites. The MCS was derived from the pET vector pET28b (Novagen).

Any ORF sequence generated by PCR or other means can be cloned into the MCS in such a way as to allow in frame fusion of the inserted ORF with the start codon.

In the invention, three cloning vectors were used which are pDL242 (6.3 kbp) (Figure 2), pDL243 (6.3 kbp) (Figure 3) and pDG364 that carry the Prrn0-RBS(sspA)-MCS cassette.

pDL242 (Figure 2) is derived from the plasmid pDG1663 (Guerout-Fleury et al., 1996). pDG1663 allows insertion of foreign DNA into the B. subtilis chromosome by what is referred to as a double crossover recombination or marker replacement as shown in Figure 4 and described in (Guerout-Fleury et al., 1996). Integration occurs only at the thrC locus. pDL242 carries the erm gene that renders transformed cells resistant to erythromycin.

To use this plasmid, a DNA is inserted at the MCS site of pDL242. Ligated molecules are transformed into E. coli with selection for Amp® (ampicillin resistance) and recombinants are screened using PCR analysis of plasmids. Plasmid clones are then prepared in Escherichia coli and plasmid DNA clone verified by sequencing across the site of the fusion junctions. Next, the plasmid is linearised by restriction digest using sites
cutting in the backbone of pDL242 (see (Guerout-Fleury et al., 1996)) and DNA transformed into competent *B. subtilis* with selection for Erm\(^8\). PvuI is the preferred enzyme for linearisation of pDL242. Transformants can only arise if a double crossover recombination has occurred between homologous segments of the *thrC* gene carried on the host chromosome and the pDL242 clone.

Transformants are checked to ensure they are Erm\(^8\) and ThrC\(^-\) (since integration at the *thrC* locus will destroy the gene destroying the ability of cells to grow without added threonine).

Cells are then cultured and expression of the gene product cloned into the cassette verified by Western blotting, dot-blotting quantification and immunofluorescence microscopy.

pDL243 is shown in Figure 3 and is similar to pDL242. The vector is derived from the plasmid pDG364 (Cutting and Vander-Horn, 1990; Karmazyn-Campelli et al., 1992). pDG364 allows insertion of foreign DNA into the *B. subtilis* chromosome by what is referred to as a double crossover recombination or marker replacement as shown in Figure 5 and described in (Cutting and Vander-Horn, 1990; Guerout-Fleury et al., 1996). Integration occurs only at the *amyE* locus. pDL243 carries the *cat* gene that renders transformed cells resistant to chloramphenicol.

To use this plasmid, a DNA is inserted at the MCS site of pDL243. Ligated molecules are transformed into *E. coli* with selection for Amp\(^8\) (ampicillin resistance) and recombinants are screened using PCR analysis of plasmids. Plasmid clones are then prepared in *Escherichia coli* and plasmid DNA clone verified by sequencing across the site of the fusion junctions. Next, the plasmid is linearised by restriction digest using sites cutting in the backbone of pDL243 (see (Cutting and Vander-Horn,
1990)) and DNA transformed into competent B. subtilis with selection for Cm^8. PvuII is the preferred enzyme for linearisation of pDL243. Transformants can only arise if a double crossover recombination has occurred between homologous segments of the amyE gene carried on the host chromosome and the pDL243 clone as shown in Figure 5.

Transformants are checked to ensure they are Cm^8 and AmyE^- (since integration at the amyE locus will destroy the gene destroying the ability of cells to grow without added threonine).

Cells are then cultured and expression of the gene product cloned into the cassette verified by Western blotting, dot-blotting quantification and immunofluorescence microscopy.

Regarding the translational start signals (RBS = ribosome binding site or Shine Dalgarno (SD) sequence, the optimum rbs is AAAGAGGTTGA and the sspA RBS has AAGGAGGTTGA. In principle the rbs could be taken from any gene or made synthetically.

The pharmaceutical composition according to the invention comprises a spore according to the invention and a pharmaceutically acceptable carrier and/or excipient. Processes for manufacturing a pharmaceutical composition are well known. The components of the composition may be combined with any combination of optional additives (e.g., at least one diluent, binder, excipient, stabilizer, dessicant, preservative, coloring, or combinations thereof). See, generally, Ullmann’s Encyclopedia of Industrial Chemistry, 6th Ed (electronic edition, 1998); Remington's Pharmaceutical Sciences, 22nd (Gennaro, 1990, Mack Publishing); Pharmaceutical Dosage Forms, 2nd Ed. (various editors, 1989-1998, Marcel Dekker); and Pharmaceutical Dosage Forms and Drug Delivery Systems (Ansel et al., 1994, Williams & Wilkins).
A pharmaceutical composition according to the invention may be in the form of an cream, emulsion, gel, lotion, ointment, paste, solution, suspension, or other liquid forms known in the art. A pharmaceutical composition according to the invention may optionally also comprise an adjuvant which potentiates an antigen-specific immune response.

A sterile liquid composition suitable for use as the pharmaceutical composition according to the invention may be prepared by suspending an intended component of the formulation in a sufficient amount of an appropriate sterile solvent. Generally, dispersions are prepared by incorporating the various sterilized components of the formulation into a sterile vehicle which contains the basic dispersion medium. For production of solid forms that are required to be sterile, vacuum drying or freeze drying can be used. Solid dosage forms (e.g., powders, granules, pellets, tablets) or liquid dosage forms (e.g., liquid in ampules, capsules, vials) can be made from at least one active ingredient or component of the formulation.

The relative amounts of active ingredients within a dose and the dosing schedule may be adjusted appropriately for efficacious administration to a subject (e.g., animal or human). This adjustment may depend on the subject's particular disease or condition, and whether therapy or prophylaxis is intended. To simplify administration of the formulation to the subject, each unit dose would contain the active ingredients in predetermined amounts for a single round of immunization.

There are numerous causes of protein instability or degradation, including hydrolysis and denaturation. In the case of denaturation, a protein's conformation is disturbed and the protein may unfold from its usual globular structure. Rather than refolding to its natural conformation,
hydrophobic interaction may cause clumping of molecules together (i.e., aggregation) or refolding to an unnatural conformation. Either of these results may entail diminution or loss of antigenic activity. A stabilizer may be added to lessen or prevent such problems.

The pharmaceutical composition according to the invention, or any intermediate in its production, may be pretreated with protective agents (i.e., cryoprotectants and dry stabilizers) and then subjected to cooling rates and final temperatures that minimize ice crystal formation. By proper selection of cryoprotective agents and the use of preselected drying parameters, almost any formulation might be cryoprepared for a suitable desired end use.

It should be understood in the following discussion of optional additives like excipients, stabilizers, dessicants, and preservatives are described by their function. Thus, a particular chemical may act as some combination of excipient, stabilizer, dessicant, and/or preservative. Such chemicals would be considered immunologically inactive because it does not directly induce an immune response, but it increases the response by enhancing immunological activity of the antigen or adjuvant: for example, by reducing modification of the antigen or adjuvant, or denaturation during drying and dissolving cycles.

Stabilizers include cyclodextrin and derivatives thereof (see U. S. Patent 5,730,969). Suitable preservatives such as sucrose, mannitol, sorbitol, trehalose, dextran, and glycerin can also be added to stabilize the final formulation. A stabilizer selected from nonionic surfactants, D-glucose, D-galactose, D-xylose, D-glucuronic acid, salts of D-glucuronic acid, trehalose, dextrans, hydroxyethyl starches, and mixtures thereof may be added to the formulation. Addition of an alkali metal salt or magnesium chloride may stabilize a polypeptide, optionally including serum albumin
and freeze-drying to further enhance stability. A polypeptide may also be stabilized by contacting it with a saccharide selected from the group consisting of dextran, chondroitin sulfuric acid, starch, glycogen, insulin, dextrin, and alginic acid salt. Other sugars that can be added include monosaccharides, disaccharides, sugar alcohols, and mixtures thereof (e.g., glucose, mannose, galactose, fructose, sucrose, maltose, lactose, mannitol, xylitol). Polyols may stabilize a polypeptide, and are water-miscible or water-soluble. Suitable polyols may be polyhydroxy alcohols, monosaccharides and disaccharides including mannitol, glycerol, ethylene glycol, propylene glycol, trimethyl glycol, vinyl pyrrolidone, glucose, fructose, arabinose, mannose, maltose, sucrose, and polymers thereof. Various excipients may also stabilize polypeptides, including serum albumin, amino acids, heparin, fatty acids and phospholipids, surfactants, metals, polyols, reducing agents, metal cheating agents, polyvinyl pyrrolidone, hydrolyzed gelatin, and ammonium sulfate.

Single-dose formulations can be stabilized in poly (lactic acid) (PLA) and poly (lactide-co-glycolide) (PLGA) microspheres by suitable choice of excipient or stabilizer. Trehalose may be advantageously used as an additive because it is a nonreducing saccharide, and therefore does not cause aminocarbonyl reactions with substances bearing amino groups such as proteins.

The invention is illustrated with reference to the following Figures of the accompanying drawings:

**Figure 1** shows schematically the role of the *Bacillus anthracis* protein Protective Antigen;

**Figure 2** shows the construction of vector DL242;
Figure 3 shows the construction of vector DL243;

Figure 4 shows the integration of vector DL242 into *B. subtilis* chromosome;

Figure 5 shows the integration of vector DL243 into *B. subtilis* chromosome;

Figure 6 shows single constructs of PA63 and Domain 4 of PA with CotB and a CotB promoter;

Figure 7 shows single constructs of PA63 and Domain 4 of PA with CotC and a CotC promoter;

Figure 8 shows single constructs of PA83, PA63 and Domain 4 of PA with *rrnO* promoter;

Figure 9 shows Western blots specific for PA wherein PY79, non-recombinant *B. subtilis*. Spore coat extracts are fractionated by SDS-PAGE; arrows point to the fusion proteins CotB-PA63 (122 kDa), CotB-Domain 4 (75 kDa), CotC-PA63 (75 kDa), and CotC-Domain 4 (28 kDa), respectively; in the last 2 lanes, vegetative cell lysates are fractionated by SDS-PAGE showing PA63 (63 kDa) and Domain 4 (16 kDa) respectively;

Figure 10 shows the results from immune responses after parenteral immunisation wherein a group of mice is immunised (†) with recombinant *B. subtilis* spores expressing CotB-Domain 4, *rrnO*-PA63 (▲); CotB-Domain 4, *rrnO*-Domain 4 (■); CotC-PA63, *rrnO*-PA63 (△); and CotC-PA63, *rrnO*-Domain 4 (□); sera are tested by ELISA for PA-specific IgG and endpoint titers are
calculated as dilutions that give the same optical density (OD\textsubscript{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant \textit{B. subtilis} spore (●) are included as control groups;

**Figure 11** shows the protein sequence listing for \textit{B. anthracis} protective antigen Domain I herein referred to as SEQ ID No. 1;

**Figure 12** shows the protein sequence listing for \textit{B. anthracis} protective antigen Domain II herein referred to as SEQ ID No. 2;

**Figure 13** shows the protein sequence listing for \textit{B. anthracis} protective antigen Domain III herein referred to as SEQ ID No. 3;

**Figure 14** shows the protein sequence listing for \textit{B. anthracis} protective antigen Domain IV herein referred to as SEQ ID No. 4;

**Figure 15** shows the DNA sequence listing for promoter (\textit{rrnO}) – RBS (\textit{sspA}) – MCS herein referred to as SEQ ID No. 5;

**Figure 16** shows the protein sequence listing for \textit{B. anthracis} protective antigen PA83 herein referred to as SEQ ID No. 6;

**Figure 17** shows the protein sequence listing for \textit{B. anthracis} protective antigen PA63 herein referred to as SEQ ID No. 7;

**Figure 18** shows the results from immune responses after intraperitoneal immunisation wherein a group of mice is immunised (↑) with recombinant \textit{B. subtilis} expressing the stated fragments of anthrax protective antigen in a vegetative cell state; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as
dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (♀) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

**Figure 19** shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in Figure 18; at day 45 the ELISA and TNA titres of the final sera were measured;

**Figure 20** shows the results from immune responses after nasal immunisation wherein a group of mice is immunised (†) with recombinant *B. subtilis* expressing the stated fragments of anthrax protective antigen in a vegetative cell state; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD_{450nm}) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (♀) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

**Figure 21** shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in Figure 20; at day 69 the ELISA and TNA titres of the final sera were measured;

**Figure 22** shows the results from immune responses after subcutaneous immunisation wherein a group of mice is immunised (†) with recombinant *B. subtilis* spores expressing the stated fragments of anthrax protective antigen on the spore coat; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD_{450nm})
as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

**Figure 23** shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in Figure 22; at day 45 the ELISA and TNA titres of the final sera were measured;

**Figure 24** shows the results from immune responses after nasal immunisation wherein a group of mice is immunised (↑) with recombinant *B. subtilis* spores expressing the stated fragments of anthrax protective antigen on the spore coat; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD<sub>450nm</sub>) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

**Figure 25** shows the results from immune responses after oral immunisation wherein a group of mice is immunised (↑) with recombinant *B. subtilis* expressing the stated fragments of anthrax protective antigen in the vegetative cell state; sera are tested by ELISA for PA-specific IgG and endpoint titers are calculated as dilutions that give the same optical density (OD<sub>450nm</sub>) as 1/40 dilution of a pooled pre-immune sample; naïve, non-immunised (○) and mice immunised with non-recombinant *B. subtilis* spore (●) are included as control groups;

**Figure 26** shows the results from immune responses of the group of mice tested in the experiments for which the data is shown in
Figure 25; at day 69 the ELISA and TNA titres of the final sera were measured;

Figure 27 shows gene fusions in plasmid pDG364;

Figure 28 shows the DNA sequence listing for Anthrax Protective Antigen PA63 herein referred to as SEQ ID No. 8;

Figure 29 shows the DNA sequence listing for Anthrax Protective Antigen Domain IV herein referred to as SEQ ID No. 9;

Figure 30 shows the DNA sequence listing for Anthrax Protective Antigen Domain DIb23 herein referred to as SEQ ID No. 10;

Figure 31 shows the protein sequence listing for Anthrax Protective Antigen Domain DIb23 herein referred to as SEQ ID No. 11;

Figure 32 shows the DNA sequence listing for B. subtilis CotB protein from residue -263 to residue +825 herein referred to as SEQ ID No. 12;

Figure 33 shows the protein sequence listing for B. subtilis CotB protein from residue -263 to residue +825 herein referred to as SEQ ID No. 13;

Figure 34 shows the DNA sequence listing for B. subtilis CotC protein from residue -179 to residue +198 herein referred to as SEQ ID No. 14;
Figure 35 shows the protein sequence listing for B. subtilis CotC protein from residue -179 to residue +198 herein referred to as SEQ ID No. 15;

Figure 36 shows single constructs of PA83 (full length), PA83, PagD1b23, PA63 and PagD4 with the rrnO promoter in pDL242;

Figure 37 shows the DNA sequence listing for Anthrax PA83 (full length) herein referred to as SEQ ID No. 16; and

Figure 38 shows the DNA sequence listing for Anthrax PA83 herein referred to as SEQ ID No. 17.

Figure 1 illustrates the role of PA. In the first step, PA is secreted from the B. anthracis cell. PA is herein referred to as PA83 (full length) and is SEQ ID No. 16. Secretion cleaves the first 29 amino acids of PA83 (full length) to produce the mature PA (PA83). PA83 (735 amino acids) carries 4 domains which are:

Domain 1 (residues 1-250) which is SEQ ID No. 1;
Domain 2 (residues 251-487) which is SEQ ID No. 2;
Domain 3 (488-594) which is SEQ ID No. 3; and
Domain 4 (residues 595-735) which is SEQ ID No. 4.

Domain 4, covering residues 595-735 of the C-terminus of PA is required for receptor binding and monoclonal antibodies specific to this region can block receptor binding.

In the second step of the process illustrated in Figure 1, mature PA83 binds to cell receptor (using domain 4) and is cleaved (at domain 1) by a furin-like protease to free PA20 (subdomain 1a) and expose the EF/LF binding site (in subdomain 1b). The activated PA63s (using domains 2
and 3) heptamerise (Milne et al., 1994), and synchronously bind to EF/LF (up to 3 molecules EF/LF per PA63 heptamer), (Mogridge et al., 2002). The toxin complex is internalised by receptor-mediated endocytosis (Gordon et al., 1988). When the endosome fuses to an acidic compartment, low pH enables the formation of a pore (using domain 2) through the lipid membrane (Blaustein et al., 1989; Koehler and Collier, 1991; Menard et al., 1996; Milne and Collier, 1993) hence the translocation of EF/LF moieties into the cytoplasm. The outcome is then cell death.

The invention will now be illustrated with reference to the following examples which are not intended to limit the scope of the invention claimed.

**EXAMPLE 1**

The Prrn0-RBS(sspA)-MCS vector was constructed as follows:

*rrnO* promoter (290 bp) was amplified from *B. subtilis* chromosome (PY79) with forward (F) primer containing a BgIII site, and reverse (R) primer containing a XbaI site.

*sspA* ribosome binding site (RBS) of *B. subtilis* (20 bp) was synthesised by annealing 2 oligonucleotides so that the double stranded DNA contains 2 cohesive ends, XbaI at 5' and NcoI at 3'.

These 2 fragments were cloned into pET28b restricted with BgIII and NcoI.
**EXAMPLE 2**

Construction of Recombinant *B. subtilis* Strains

The non-pathogenic spore-forming bacterium *Bacillus subtilis* was engineered to express different domains of the protective antigen (PA) from *Bacillus anthracis*.

The domains chosen were: mature secreted PA83 (735 aa, 82.7 kDa), functional PA63 (568 aa, 63.5 kDa), and Domain 4 of PA (141 aa, 16.1 kDa).

The ways in which these antigens were displayed are: in-frame fusion with the *B. subtilis* spore coat (*cotB* and *cotC*) proteins for spore coat expression, or under the constitutive ribosomal RNA promoter (*rrnO*) for vegetative cell expression. Briefly, the fusion recombinant DNA (*cotB/cotC* with their promoters – antigens, or *rrnO* promoter – antigens) are introduced into *B. subtilis* chromosome by double-crossover integration at the *amyE* or *thrC* loci. The constructs (recombinant *B.*
subtilis expressing heterologous antigens) are selected by means of antibiotic markers (Figure 4 and 5).

The single constructs (spores expressing one antigen) are shown in Figures 6 and 7:
1. CotB-PA63
2. CotB-Domain 4
3. CotC-PA63
4. CotC-Domain 4

The following constructs are shown in Figure 8:
5. rrnO-PA83
6. rrnO-PA63
7. rrnO-Domain 4

The following double constructs (spores expressing two antigens) were also used:
9. CotB-Domain 4, rrnO-PA83 (2 and 5)
10. CotB-Domain 4, rrnO-PA63 (2 and 6)
11. CotB-Domain 4, rrnO-Domain 4 (2 and 7)
12. CotB-Domain 4, rrnO-sLTB (2 and 8)
13. CotC-PA63, rrnO-PA83 (3 and 5)
14. CotC-PA63, rrnO-PA63 (3 and 6)
15. CotC-PA63, rrnO-Domain 4 (3 and 7)
16. CotC-PA63, rrnO-sLTB (3 and 8)

Expression of PA on spore coat or in vegetative cells is checked by Western blots (Figure 9) and confocal immunofluorescent microscopy.

EXAMPLE 3
Evaluation of Immune Responses

With the constructs of Example 2, groups of 8 A/J inbred mice were immunised by different routes: intra-peritoneal (i.p.), oral, and intra-nasal (i.n.).

Parenteral immunisation (Figure 10)
Intra-peritoneal injection utilised 3 doses of 1x10⁸ spores on days 0, 20 and 40. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 55. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 10). Control groups were non-immunised (naïve), or immunised with non-recombinant spores, or with purified PA protein. This study reveals constructs that are most immunogenic (titers > 2,000) and pilots the mucosal immunisations.

Mucosal immunisations
Mucosal immunisations utilise 2 routes. Mice were dosed orally with 1x10¹⁰ spores, or intra-nasally with 1x10⁹ spores on days 0, 20 and 40. Control groups are included as in the i.p. route. The immune responses were assessed by various methods. Anti-PA serum IgG and its subclass (IgG1, IgG2a, IgG2b, IgG3) titres were determined by ELISA, so were fecal secreted IgA (for oral) and saliva IgA (for i.n.) titres on day 55. The results show the type of immune responses to PA expressed in B. subtilis mucosally administered to mice. The spore-specific responses were examined to further understand the nature of immunogenicity when using B. subtilis spores as mucosal delivery vehicles of heterologous antigens.

Preliminary results show sero-conversion by the nasal and oral routes.

EXAMPLE 4
Construction of further Recombinant *B. subtilis* Strains

The non-pathogenic spore-forming bacterium *Bacillus subtilis* was engineered to express different domains of the protective antigen (PA) from *Bacillus anthracis*.

The domains chosen were: PA83 (full length, 764aa), mature secreted PA83 (735 aa, 82.7 kDa), PagD1b23 (47kDa, domain D1b23), functional PA63 (568 aa, 63.5 kDa), Domain 4 of PA (also herein referred to as PagD4, 141 aa, 16.1 kDa).

The ways in which these antigens were displayed are: in-frame fusion with the *B. subtilis* spore coat (*cotB* and *cotC*) proteins for spore coat expression, or under the constitutive ribosomal RNA promoter (*rrnO*) for vegetative cell expression. Briefly, the fusion recombinant DNA (*cotB/cotC* with their promoters – antigens, or *rrnO* promoter – antigens) are introduced into *B. subtilis* chromosome by double-crossover integration at the *amyE* or *thrC* loci. The constructs (recombinant *B. subtilis* expressing heterologous antigens) are selected by means of antibiotic markers (Figure 4 and 5).

The following constructs in plasmid pDG364 are shown in Figure 27:
1. CotB-PA63
2. CotB-PagD4
3. CotC-PagD4

The following constructs in plasmid pDL242 are shown in Figure 37:
4. *rrnO*-PA83 (full length)
5. *rrnO*-PA83
6. *rrnO*-PagD1b23
7. rrnO-PA63
8. rrnO-PagD4

The spores transformed by construct 4 of this Example are referred to as PA83 sec. This is because in the vegetative cell state the PA83 antigen is secreted from the cell. The PA83 (full length) protein which is expressed by the construct includes a 29 amino acid leader sequence which enables secretion. This leader sequence is chopped off the protein as it is translocated across a cell membrane and released from the cell. Thus the secreted polypeptide is PA83, not PA83 (full length).

The spores transformed by construct 5 and 7 of this Example are referred to as PA83 intra and PA63 intra because the antigen is not secreted from the cell; instead it is only available intra-cellularly.

The following spores were also used:

9. CotC-PagD4 Domain 4 of PA fused to the CotC spore coat protein
10. CotB-PagD4 Domain 4 of PA fused to CotB spore coat protein
11. CotB-PagD1b23 Domain D1b23 fused to CotB spore coat protein

For the CotB/C constructs, the initial fusion of gene sequences are made in E. coli and then subcloned into pDG364 (using a MCS). Next, pDG364 is linearised and introduced into B. subtilis cells by DNA mediated transformation as shown in Figure 5.

For rrnO constructs that permit vegetative gene expression, the relevant gene sequence is cloned into the MCS (multiple cloning site) of pDL242 (Figure 2) to allow fusion to the rbs of the sspA gene under the control of
the PrrnO promoter. It is noted that rrnO is a gene only expressed in vegetative cells, that is, only in the germinating/germinated spore. Coat proteins (i.e., CotC and/or CotB are surface exposed proteins on the spore.

The pDL242 recombinant plasmid is then linearised and introduced into B. subtilis cells by DNA mediated transformation as shown in Figure 4.

EXAMPLE 5

Evaluation of Immune Responses

With the constructs of Example 4, groups of 6 Balb/C inbred mice were immunised by different routes: intra-peritoneal (i.p.), subcutaneous, intra-nasal (i.n.) and oral.

Intraperitoneal immunisation (Figures 18 and 19)

Intra-peritoneal injection utilised 3 doses of 1x10⁹ spores on days 0, 16 and 29. The spores had been transformed with vegetative cell expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 45. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 18) and using the TNA assay of Example 7 (Figure 19). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5µg/dose), or with a 100 µl/dose (which is one fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Human anthrax vaccine is a cell-free extract of B. anthracis culture medium (Sterne strain). The medium extract contains unknown amount of PA (mainly) and LF/EF (fraction) and other secreted proteins of the B.
anthracis strain. The extract is absorbed with Alum (aluminium hydroxide/phosphate).

Nasal immunisation (Figures 20 and 21)
Nasal injection utilised 51 doses of 2x10⁸ spores on each day from day 0 to day 50. The spores had been transformed with vegetative cell expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 69. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 20) and using the TNA assay of Example 7 (Figure 21). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with a 20 μl/dose (which is one twenty fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Subcutaneous immunisation (Figures 22 and 23)
Subcutaneous injection utilised 3 doses of 1x10⁸ spores on days 0, 16 and 29. The spores had been transformed with spore coat expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 45. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 22) and using the TNA assay of Example 7 (Figure 23). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with 100 μl/dose (which is one fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

Nasal immunisation (Figure 24)
Nasal injection utilised 51 doses of 2x10⁸ spores on each day from day 0 to day 50. The spores had been transformed with spore coat expression
constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 69. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 24). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with 100 μl/dose (which is one fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

**Oral immunisation (Figures 25 and 26)**

Oral injection utilised 7 doses of 1x10⁶ spores on days 1, 2, 3, 21, 22, 35 and 36. The spores had been transformed with vegetative cell expression constructs. Serum samples were taken one day prior to an immunisation, and mice were sacrificed on day 69. The humoral immune responses to PA via serum IgG titers were evaluated by ELISA (Figure 25) and using the TNA assay of Example 7 (Figure 26). Control groups were non-immunised (naïve), or immunised with non-recombinant spores (data labelled as PY29), or with purified PA protein (5μg/dose), or with a 20 μl/dose (which is one twenty fifth of a human dose) of human anthrax vaccine (labelled as AVA) as an internal control.

**EXAMPLE 6**

The following methodology was used in the anti-PA ELISA assay.

Plates were coated with 50 μl/well of purified protective antigen (1 μg/ml in PBS) and left at room temperature overnight. After blocking with 2% BSA in PBS for 1.5 h at 37°C serum samples were applied using a 2-fold dilution series starting with a 1/40 dilution in ELISA diluent buffer (0.1M Tris-HCl, pH 7.4; 3% (w/v) NaCl; 2% (w/v) BSA; 10% (v/v) fetal bovine serum (Sigma); 0.1% (v/v) Triton-X-100; 0.05% (v/v) Tween-20). Every plate carried replicate wells of a negative control (a 1/40 diluted
pre-immune serum), a positive control (serum from mice immunised parentally with protective antigen). Plates were incubated for 1 h at 37°C before addition of anti-mouse HRP conjugate (Sigma). Plates were incubated for a further 1 h at 37°C then developed using the substrate TMB (3, 3', 5, 5'-tetramethyl-benzidine; Sigma). Reactions were stopped using 2M H$_2$SO$_4$. Dilution curves were drawn for each sample and endpoint titres calculated as the dilution producing the same optical density as the 1/40 dilution of a pooled pre-immune serum.

EXAMPLE 7

The following methodology was used in the Toxin Neutralisation Assay (TNA).

The murine macrophage-like cell line RAW264.7 (obtained from the European Collection of Animal Cell Cultures [ECACC]) was cultured as monolayers in DMEM medium (Sigma) supplemented with 10% (v/v) fecal bovine serum, 50 µg ml$^{-1}$ penicillin and 50 µg ml$^{-1}$ streptomycin, in an atmosphere of 90% humidity containing 5% CO$_2$ at 37°C. One day before use, the cells were detached by gentle scraping and seeded into 96-multiwell disposable plates in the same medium with 0.1 mM HEPES at a density of approximately 1 x 10$^6$ cells per well. Test sera were serially diluted in DMEM/HEPES medium, and mixed with anthrax lethal toxin (0.1 µg/ml LF, 0.08 µg/ml PA [Quadratic Diagnostics]) with volume ratio 1:1 in a separate 96-well plate. After 1 h incubation at 37°C, corresponding wells were transferred to the macrophage culture plate. After 3 h incubation at 37°C, surviving macrophages were measured by addition of WST-1 reagent (Roche) and further incubation for 4 h at 37°C. The absorbance was read at 450 nm wavelength, and results were scored against positive (medium only) and negative (toxin only) controls.
References


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CLAIMS

1. A non-pathogenic spore comprising an antigenic fragment of anthrax protective antigen.

2. A spore as defined in claim 1 which is a Bacillus species spore; preferably a spore from one or more of the following organisms: *Bacillus alvei; Bacillus badius; Bacillus brevis; Bacillus cereus; Bacillus coagulans; Bacillus fastidiosus; Bacillus licheniformis; Bacillus mycoides; Bacillus pasteurii; Bacillus sphaericus; Bacillus aneurinolyticus; Bacillus carotarum; Bacillus flexus; Bacillus freudenreichii; Bacillus macroides; Bacillus similibadius; Bacillus thiaminolyticus; Bacillus subtilis; Bacillus pumilus; Bacillus vallismortis; Bacillus bengalicus; Bacillus flexus; and/or Bacillus licheniformis; more preferably a spore from *Bacillus subtilis*.

3. A spore as defined in claim 1 or claim 2 which comprises an antigenic fragment of anthrax protective antigen in the form of a protein attached to the proteinaceous coat of the spore.

4. A spore as defined in any one of the preceding claims which comprises an antigenic fragment of anthrax protective antigen encoded in the form of DNA which is adapted to be expressed when the spore germinates.

5. A spore as defined in any one of the preceding claims wherein the antigenic fragment is one or more of:
   - PA83 which is SEQ ID No. 6;
   - PA63 which is SEQ ID No. 7;
   - Domain 1 of the protective antigen which is SEQ ID No. 1;
   - Domain 2 of the protective antigen which is SEQ ID No. 2;
Domain 3 of the protective antigen which is SEQ ID No. 3;
Domain 4 (residues 595-735) of the protective antigen which is
SEQ ID No. 4; and
Domain D1b23 which is SEQ ID No. 10.

6. A spore substantially as hereinbefore described.

7. A spore as defined in any one of the preceding claims which is for
use as an anthrax vaccine.

8. A pharmaceutical composition comprising a non-pathogenic spore
comprising at least an antigenic fragment of anthrax protective antigen in
association with a pharmaceutically acceptable carrier and/or excipient.

9. A composition as defined in claim 8 wherein the spore is a Bacillus
species spore; preferably a spore from one or more of the following
organisms: Bacillus alvei; Bacillus badius; Bacillus brevis; Bacillus
cereus; Bacillus coagulans; Bacillus fastidiosus; Bacillus licheniformis;
Bacillus mycoides; Bacillus pasteuri; Bacillus sphaericus; Bacillus
aneurinolyticus; Bacillus carotarum; Bacillus flexus; Bacillus
freudenreichi; Bacillus macroides; Bacillus similibadius; Bacillus
thiaminolyticus; Bacillus subtilis; Bacillus pumilus; Bacillus vallismortis;
Bacillus bengalicus; Bacillus flexus; and/or Bacillus licheniformis; more
preferably a spore from Bacillus subtilis.

10. A composition as defined in claim 8 or claim 9 wherein the spore
comprises at least an antigenic fragment of anthrax protective antigen in
the form of a protein attached to the proteinaceous coat of the spore.

11. A composition as defined in any one of claims 8 to 10 wherein the
spore comprises at least an antigenic fragment of anthrax protective
antigen encoded in the form of DNA which is adapted to be expressed when the spore germinates.

12. A composition as defined in any one of claims 8 to 11 which comprises an antigenic fragment of anthrax protective antigen, preferably the fragment is one or more of:
   PA83 which is SEQ ID No. 6;
   PA63 which is SEQ ID No. 7;
   Domain 1 of the protective antigen which is SEQ ID No. 1;
   Domain 2 of the protective antigen which is SEQ ID No. 2;
   Domain 3 of the protective antigen which is SEQ ID No. 3;
   Domain 4 (residues 595-735) of the protective antigen which is SEQ ID No. 4; and
   Domain D1b23 which is SEQ ID No. 10.

13. A composition as defined in any one of claims 8 to 12 which further comprises an adjuvant which potentiates an antigen-specific immune response.

14. A composition as defined in any one of claims 8 to 13 wherein the spore is substantially as hereinbefore described.

15. A composition as defined in any one of claims 8 to 14 for use as an anthrax vaccine.

16. Use of a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen in the manufacture of a vaccine for anthrax for non-parenteral administration, preferably for nasal and/or oral administration.
17. Use according to claim 16 wherein the spore is as defined in any one of claims 9 to 12.

18. Use of a composition as defined in any one of claims 8 to 15 in the manufacture of a vaccine for anthrax.

19. Use of a spore as defined in any one of claims 1 to 7 in the manufacture of a vaccine for anthrax.

20. Use as defined in claim 18 or claim 19 wherein the vaccine is for non-parenteral administration, preferably for nasal and/or oral administration.

21. A method of inducing immunity to anthrax in a mammal susceptible to anthrax infection which method comprises non-parenteral administration, preferably nasal or oral administration of an effective amount of a vaccine comprising a non-pathogenic spore comprising at least an antigenic fragment of anthrax protective antigen.

22. A method as defined in claim 21 wherein the spore is as defined in any one of claims 9 to 12.

23. A method of inducing immunity to anthrax in a mammal susceptible to anthrax infection which method comprises administration of an effective amount of a vaccine comprising a spore as defined in any one of claims 1 to 7 or of a composition as defined in any one of claims 8 to 15.

24. A method as defined in claim 23 wherein the vaccine is for non-parenteral administration, preferably for nasal and/or oral administration.
Figure 1
Figure 2

DL242

TTGACC  TACTAT  ACAAGGAGGTGAGACCCATG
-35       -10       +1       RBS       Met       MCS

thrC front         erm         thrC back

Figure 3

DL243

TTGACC  TACTAT  ACAAGGAGGTGAGACCCATG
-35       -10       +1       RBS       Met       MCS

amyE front         cat         amyE back
Figure 5
Figure 9
Figure 10
Figure 11

Figure 12

Figure 13

Figure 14
Figure 15

Figure 16

Figure 17
Figure 18

ELISA titers vs time for Intraperitoneal Immunisation with vegetative cell expression constructs
ELISA and TNA titers of final sera (Day 45) for Intraperitoneal Immunisation with vegetative cell expression constructs

Figure 19
ELISA titers vs time for Nasal Immunisation with vegetative cell expression constructs

Figure 20
ELISA and TNA titers of final sera (Day 69) for Nasal Immunisation with vegetative cell expression constructs

Figure 21
ELISA titers vs time for Subcutaneous Immunisation with spore coat expression constructs

Figure 22
ELISA and TNA titers of final sera (Day 45) for Subcutaneous Immunisation with spore coat expression constructs

Figure 23
ELISA titers vs time for Nasal Immunisation with spore coat expression constructs

Figure 24
ELISA titers vs time for Oral Immunisation with vegetative cell expression constructs

Figure 25
ELISA and TNA titers of final sera (Day 69) for Oral Immunisation with vegetative cell expression constructs

Figure 26
Figure 27
Figure 28
Figure 31

acggattagccgtttgtcctcatggacccgtataaaaaagaatgatatttga
gcttttgaccgctgagcggcgtatgtatctcgagttatatttataaaaaaccgtttac
ggttttaaagttaatttttttttttttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
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Royal Holloway University of London

Improvements in or relating to Vaccination

JPP237

17

PatentIn version 3.3

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250

PRT

Bacillus anthracis

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Page 17
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/32 A61K39/07

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, BIOSIS, MEDLINE, EMBASE, WPI Data, PAJ, LIFESCIENCES, SCISEARCH, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>BROSSIER FABIEN ET AL: &quot;Anthrax spores make an essential contribution to vaccine efficacy&quot; INFECTION AND IMMUNITY, vol. 70, no. 2, February 2002 (2002-02), pages 661-664, XP002324746 ISSN: 0019-9567 abstract page 661 page 662, column 1, paragraphs 1,3 _<em><strong>/</strong></em></td>
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Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents:
- **A**: document defining the general state of the art which is not considered to be of particular relevance
- **E**: earlier document but published on or after the international filing date
- **L**: document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- **O**: document referring to an oral disclosure, use, exhibition or other means
- **P**: document published prior to the international filing date but later than the priority date claimed

*I* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**X**: document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**Y**: document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

*F* document member of the same patent family

Date of the actual completion of the international search: 15 April 2005

Date of mailing of the international search report: 02/05/2005

Name and mailing address of the ISA:
European Patent Office, P.O. 5816 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3018

Authorized officer: Voigt-Ritzer, H
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**INTERNATIONAL SEARCH REPORT**

**Box II  Observations where certain claims were found unsearchable (Continuation of Item 2 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.: 21–24 because they relate to subject matter not required to be searched by this Authority, namely:
   
   Although claims 21–24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. **☐** Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. **☐** Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box III  Observations where unity of invention is lacking (Continuation of Item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. **☐** As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. **☐** As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. **☐** As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:  

4. **☐** No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant’s protest.

☐ No protest accompanied the payment of additional search fees.
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