

**Identification of structural and antigenic components of *Bacillus anthracis* spores and their application to rapid detection using biosensors**

By

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## **Abstract**

The work presented in this thesis describes a rational design approach to the rapid detection of *Bacillus anthracis* spores. A range of assay formats are available for detection of *B. anthracis*, however none fulfil the criteria of speed, sensitivity or specificity. Optical biosensors have shown promise for the near real time detection of low molecular weight analytes, although exploitation of these systems for bacterial detection has been restricted due to a comparative lack of sensitivity. Electroporation was unsuitable as a disruption method for immunoassay based techniques, whereas preliminary experiments suggested sonication could be utilised. A novel tubular sonicator designed to operate in a continuous flow format, and to minimise antigen denaturation was shown to remove the exosporium and some of the underlying spore coat of *B. anthracis* spores, resulting in a significant increase in assay sensitivity (up to 500 fold). A range of methods were employed to characterise the structural and antigenic components of the spore, focusing on those released by sonication. Many of these were not suitable for detection purposes due to the high levels of identity with homologous proteins within closely related species. However, BxpA and EA1 and two novel *B. anthracis* spore proteins, a homologues of *B. cereus* ExsA and MntA, were identified as potential targets. Computer modelling techniques mapped the unique regions of these proteins to potential binding pockets, indicating that they could be utilised for the production of specific recognition elements. Due to the inherent problems with the use of traditional antibodies, single chain antibodies (scFv) were developed. EA1 was used as a target as it was consistently identified using a range of methods. Results indicated that it was predominantly associated with the spore and was not completely removed by Urografin purification and subsequent wash steps, in variance to previous work. Elimination of previously observed cross reactivity of scFv produced using a standard biopanning procedure was possible through the development of a one step competitive panning strategy. Finally, through the use of the scFv produced, combined with flow through sonication prior to the assay, near real time specific detection of *B. anthracis* spores was demonstrated through the use of a known protein target.

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## Abbreviations

The abbreviations used for amino acids and nucleotides are standard.

A <sub>x</sub>	Absorbance at X nanometres (similar notations for different wavelengths)
Aa	Amino acid
ABTS	2'2-azino-bis(3-ethylbenzthiazoline)-6-sulphonic acid
ATR	Anthrax toxin receptor
BCA	Bicinchonic acid
BLAST	Basic logical alignment search tool
Bp	Base pairs
BSA	Bovine serum albumin
BW	Biological warfare
C-	Carboxy-
CDR(s)	Complementarity determining region(s)
Cfu(s)	Colony forming units
CMG	Capillary morphogenesis protein
CO <sub>2</sub>	Carbon dioxide
°C	Degrees centigrade
Da	Daltons
DNA	Deoxyribonucleic acid
DPA	Dipicolinic acid (2,6-pyridinecarboxylic acid)
DSTO	Defence Science Technology Organisation
dNTP	Deoxyribonucleic acid triphosphate(s)
Dstl	Defence Science and Technology Laboratories
EA1	Extractable antigen 1
ECL	Electrochemiluminescent
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EdTx	Oedema toxin
EF	Oedema factor
ELISA	Enzyme linked immunosorbent assay
EM	Electromicroscopy
γPGA	γ-linked D-glutamic acid
Fab	Fragment binding antigen
FITC	Fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography

g/l	Grams per litre
h	hour(s)
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HCl	Hydrochloric acid
H <sub>2</sub> O	water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HIS	Histidine
HPA	Health protection Agency
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
Ig	Immunoglobulin
IgG	Immunoglobulin class G
IMAC	Immobilised metal affinity chromatography
KA	Equilibrium association constant
K <sub>a</sub>	association rate constant
KD	Equilibrium dissociation constant
K <sub>d</sub>	Dissociation rate constant
kb	kilobase(s)
kHz	kilohertz
KOH	Potassium hydroxide
kDa	Kilo Dalton(s)
kV	Kilovolt
kV/cm	Kilovolt(s) per centimetre
l	Litre(s)
LB	Luria Bertani
LeTx	Lethal toxin
LF	Lethal factor
LLNL	Lawrence Livermore National Laboratories
M	molar concentration
MCR	Molar coupling ratio
mAb	Monoclonal antibody
min	minute(s)
MIR	Molar incorporation ration
mg	Milligram(s)
µg	Microgramme(s)
µg/ml	Microgrammes per millilitre

$\mu$ l	Microlitre(s)
$\mu$ m	Micrometre(s)
MgCl <sub>2</sub>	Magnesium chloride
ml	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar concentration
MPBS	Phosphate buffered saline containing milk powder
MPBST	Phosphate buffered saline containing milk powder and Tween 20
MS	Mass spectrometry
mV	Millivolts
MW	Molecular weight
MWCO	Molecular weight cut off
N-	Amino
NaCl	Sodium chloride
NHS	N-Hydroxysuccinimide
ng	Nanogram(s)
nm	Nanometres
OD <sub>x</sub>	Optical density at x nanometres (similar notations for different wavelengths)
ORF	Open reading frame
PA	Protective antigen
PAGE	Polyacrylamide gel electrophoresis
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PDB	Protein Data Bank
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline containing Tween 20
PEG	Polyethylene glycol
PHLS	Public Health Laboratory Service
PIPES	Piperazine-1,4-bis(2-ethanesulphonic acid)
pmol	Picomolar concentration
PVDF	Polyvinylidene dichloride
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
s	Second(s)

Sap	Surface array protein
SASPs	Small acid soluble proteins
scFv	Single chain antibody (Single chain fragment variable)
SCOP	Structural classification of proteins
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SOD	Superoxide dismutase
Taq	<i>Thermus aquaticus</i>
TEM	Transmission electron microscopy
TEM8	Tumour endothelial marker 8
TFA	Trifluoroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
Tween 20	Polyoxyethylene sorbitan (sorbital) monlaurate
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
V	Volts
v/v	Volume per volume
WT	Wildtype
w/v	Weight per volume

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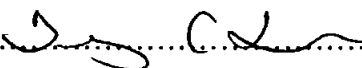
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## Chapter 1 Introduction

### 1.1 Detection of pathogenic micro organisms

Biological agents with potential use in biological warfare (BW) and bioterrorism, can broadly be divided into three categories, toxins (that have a range of sources such as animals, bacteria and plants), viruses and bacteria. The number of agents that could be utilised is extensive; however, of these a significantly smaller proportion could be effectively disseminated and infect through the aerosol route, considered the most likely route for a large scale attack (Christopher *et al.*, 1997; Eitzen, 1997; Pile *et al.*, 1998; Franz, 1997; Kortepeter and Parker, 1999). Microbial pathogens that could be used include: *Bacillus anthracis*, *Yersinia pestis*, Venezuelan equine encephalitis (VEE), *Francisella tularensis*, *Variola* virus and the haemorrhagic fever viruses (Arenaviridae, Filoviridae, Flaviviridae and Bunyaviridae) (Christopher *et al.*, 1997; Hawley and Eitzen, 2001). Toxins also represent a threat from potential use as BW agents; examples include those that could be isolated from *Clostridium botulinum*, *Ricinus communis*, trichothecane mycotoxins or staphylococcal enterotoxins (Hawley and Eitzen, 2001). Some of these agents, such as VEE, are considered incapacitating agents and others such as *Bacillus anthracis* as lethal agents (Hawley and Eitzen, 2001). Of all the lethal agents the most serious to the human host are haemorrhagic fever viruses such as Ebola, Lassa fever or Marburg as no prophylaxis or vaccines are currently available (Bray, 2003). However, these viruses are much more difficult to produce than toxins or bacteria. Virus propagation requires more sophisticated equipment and a higher degree of expertise, for example tissue culture. Bacteria by comparison are much easier to grow once a source of the pathogen has been found.

The rapid, sensitive and specific detection of pathogenic microorganisms is essential if effective countermeasures are to be provided to a susceptible population. In the case of bacteriological testing, traditional microbiology has proved a time consuming procedure. Organisms have to be isolated, grown and usually a series of biochemical tests must be completed for identification (Helrich, 1990; Poxton, 2005). Techniques such as the polymerase chain reaction (PCR) used for the amplification of pathogen-specific DNA sequences have proved to be sensitive. However, when using environmental samples a degree of sample preparation is required as impurities contained within the sample may inhibit the PCR. Furthermore the use of small sample volumes (sometimes 1  $\mu$ l) means that the sample often has to be concentrated to obtain the desired sensitivity (Radstrom *et al.*, 2004).

Biosensors are particularly attractive as a means to detect and identify potential pathogenic microorganisms due to their potential specificity and sensitivity (although this is also governed by the choice of recognition element), together with the provision of information in near real time. Biosensors also allow the analysis of complex sample matrices (Hobson *et al.*, 1996; Ivnitski *et al.*, 1999). To provide protection, i.e. timely warning of the presence of a pathogen, environmental samples are often analysed using biosensors. This presents an additional problem in that other microorganisms will also be present within the sample. The detector needs to be able to discriminate the pathogen of interest from the background and this can be achieved in a number of ways. These include (a) detection of an increase in the number of particles; (b) detection of an increase in biological particles; (c) detection of pathogenic biological agents; and (d) the specific identification of a biological agent. Specific detection is dependent on the interaction of the target analyte (for example, a protein) with a recognition element (for example, an antibody). The use of biosensors for sensitive specific detection of a pathogenic microorganism still remains a significant challenge and success is often dictated by the nature of the detection element (the specific ligand) and the choice of target analyte (Labadie and Desnier, 1992).

One bacterial agent that has attracted a significant amount of interest is *Bacillus anthracis*, the aetiological agent of anthrax. The nature of the spore and the fact that it is very closely related to other species within the *B. cereus* group makes sensitive and specific detection of this pathogenic micro-organism a particular challenge (Quinlan and Foegeding, 1997; Helgason *et al.*, 2000; Zhou *et al.*, 2002; Ivanova *et al.*, 2003; Read *et al.*, 2003; Rasko *et al.*, 2004; Todd *et al.*, 2003; Redmond *et al.*, 2004; Williams *et al.*, 2003).

## **1.2 Aims**

The aims of the work presented here are to improve our understanding of the structure and antigenic composition of the *B. anthracis* spore and associated products, and to utilise the knowledge gained for the improvement of biosensor sensitivity.

### **1.3 Objectives**

- 1) To investigate the efficacy of different methods for the disruption of *B. anthracis* spores to release putative target antigens.
- 2) To characterise surface and antigenic components of the *B. anthracis* spore.
- 3) To identify protein targets from the spore surface and assess their suitability as detection targets.
- 4) To utilise specific spore surface proteins in the production of suitable recognition elements for use within biosensors for the detection of *B. anthracis* spores.

### **1.4 Overview**

The work in this thesis describes a rational design approach to the rapid specific detection of *B. anthracis* spores. Chapter 2 is a literature review providing a background to *B. anthracis*, biosensors and recognition elements. Chapter 3 details the materials and methods used in this study. Chapter 4 evaluates and characterises disruption of the spore. Chapter 5 examines the antigenic and other surface components of the *B. anthracis* spore released by disruption and methods developed for identification of such components. Chapter 6 describes the development of a procedure for the development of specific ligands against a protein target that is also present within other *Bacillus* species. A general discussion and concluding remarks are given in Chapter 7.

## Chapter 2 Literature review

### 2.1 *Bacillus anthracis* – a bacterial pathogen with potential use in biological warfare

One bacterium of particular interest from a BW perspective is *Bacillus anthracis*, the aetiological agent of anthrax. *B. anthracis* is a Gram positive, rod shaped, non-motile, spore forming and facultatively anaerobic bacterium (Mock and Fouet, 2001). The vegetative cells have characteristic square ends when viewed under the microscope. Under aerobic conditions at the end of the exponential growth phase a centrally, or sometimes subterminally located ellipsoidal spore can be observed that does not swell the sporangium. *B. anthracis* is susceptible to a number of antibiotics such as penicillin, and other  $\beta$ -lactam agents, which is often used as a method of positive identification as other closely related species are often resistant to these antibiotics (Turnbull, 1996). Very few cases of natural antibiotic resistance to penicillin G and  $\beta$ -lactam agents have been reported (Odendaal *et al.*, 1991; Cavallo *et al.*, 2002; Mohammed *et al.*, 2002). However, a number of *in vitro* studies have demonstrated that resistance can be acquired to a range of antibiotics such as ciprofloxacin, doxycycline, fluoroquinolones and  $\beta$ -lactam antibiotics (Brook *et al.*, 2001; Price *et al.*, 2003; Athamna *et al.*, 2004).

Anthrax is recognised as being one of the earliest documented infectious diseases. In 1887 Robert Koch demonstrated that anthrax infection was due to *B. anthracis* by inoculating animals using pure culture, a model used to develop Koch's postulates. In 1881, a heat killed preparation of the organism was used as the first anthrax vaccine for livestock by Louis Pasteur (Koehler, 1999). After introduction of the vaccine and the advent of penicillin, research into this pathogenic microorganism declined as its significance as a human pathogen decreased due to the eradication of this disease in a number of countries. However, anthrax still remains enzootic in certain parts of the world (Hugh-Jones, 1999; Schmid and Kaufmann, 2002). From a military perspective, *B. anthracis* is still considered to be one of the classic BW agents. Many of the properties of *B. anthracis*, including the highly resistant nature of its endospore, ease of mass production, potential for infection via the respiratory route and proven weaponisation make, it an ideal candidate as a biological warfare agent (Wyatt, 1980; Manchee *et al.*, 1981). Concerns over potential usage have been highlighted by incidents such as the release of *B. anthracis* spores at Sverdlovsk, Russia, in 1979, the unsuccessful release by the Aum Shinrikyo cult in Japan, concerns of production and weaponisation in Iraq, and its potential usage in the Gulf wars (Zilinskas, 1997; Kortepeter and Parker, 1999). The only known deployment of *B. anthracis* as a

biological warfare agent was in the 1940s by the Japanese army in Manchuria (Harris *et al.*, 1994). More recently, the intentional release of anthrax spores from bioterrorist attacks in the USA in 2001 associated with the presentation of 22 individuals with anthrax was of particular significance. In total, 11 cases were confirmed as inhalational anthrax and 11 cutaneous (7 confirmed and 4 possible). In total there were 5 fatalities, all from the inhalational form of the disease (CDC update, 2001; Jernigan *et al.*, 2002). These incidents have confirmed the relative ease with which *B. anthracis* spores can be effectively prepared and disseminated and have led to a significant increase in interest and research into this organism (Jernigan *et al.*, 2002; Inglesby *et al.*, 1999, 2002; Traeger *et al.*, 2002; Griffiths *et al.*, 2003). The requirement for specific and sensitive detection is due to the presentation of individuals at the early stages of infection with non-specific symptoms and the need for prompt administration of antibiotics before a lethal infection is established, particularly when infection has been acquired via the respiratory route.

## **2.2 Genetic discrimination of *B. anthracis***

*B. anthracis* is a member of the *B. cereus* group. Several other closely related species are found within this group; *B. cereus*, *B. thuringiensis*, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. medusa* (Bavykin *et al.*, 2004). Of these, the closest related species are *B. cereus* and *B. thuringiensis* which, along with *B. anthracis*, are medically and economically significant. *B. cereus* is often considered a soil dwelling opportunistic pathogen and has been implicated in rare non-lethal infections most commonly associated with food poisoning. In this instance, two manifestations of the disease can be observed, an emetic form believed to be caused by a short (~5 kDa) polypeptide, and a diarrhoeal form caused by at least five enterotoxins (Kotiranta *et al.*, 2000; Jensen *et al.*, 2003). In some cases a lethal infection similar to anthrax has been observed (Miller *et al.*, 1997; Hoffmaster *et al.*, 2004). *B. thuringiensis* also produces a series of plasmid encoded endotoxins which are toxic to certain groups of the insect population (Aronson and Shai, 2001). Genetic analysis has revealed that *B. anthracis* is very closely related to *B. cereus* and *B. thuringiensis*. Helgason *et al.* (2000) suggested that all three were in fact the same species and that *B. anthracis* and *B. thuringiensis* evolved from a common ancestral species (*B. cereus*) through the acquisition of plasmids encoding toxin genes, such as pXO1 in the case of *B. anthracis*. However, there is considerable phenotypic variation between all three; for example *B. anthracis* is non-motile. However, phenotypic variation between *B. cereus* and *B. anthracis* is thought to be due to differences in expression and

regulation rather than the loss or addition of particular genes (Ivanova *et al.*, 2003). Furthermore, *B. anthracis* is exceptionally monomorphic at the molecular level (Keim *et al.*, 1997). In contrast, *B. thuringiensis* and *B. cereus* are both highly polymorphic (Helgason *et al.*, 2000). Sequencing of the *B. cereus* 10987 genome and comparison to the *B. anthracis* genome revealed that chromosomally encoded genes were more highly conserved than those observed in *B. cereus* 14579 (Rasko *et al.*, 2004). This strain was also found to contain a number of known virulence factors such as the genes encoding the NHE complex, the two major phospholipases and cytotoxin K. Furthermore, an ~209 kb plasmid (pBc10987) was also identified similar in organisation, and encoded genes, to *B. anthracis* pXO1, excluding the presence of a pathogenicity island and in particular the genes encoding toxin subunits, lethal Factor (LF), oedema factor (LF) and protective antigen (PA). A strain of *B. cereus* has also been identified that is capable of causing an illness with clinical manifestations identical to those observed with inhalational anthrax infection. This strain (G9241) was found to carry a plasmid with 99.6% identity to pXO1, including the toxin genes and a plasmid encoding a capsule that was not identical to pXO2. The presence of the pXO1-like plasmid in this strain of *B. cereus* suggests that horizontal transfer of genetically mobile elements is possible in nature (Hoffmaster *et al.*, 2004).

### **2.3 Anthrax infection**

*B. anthracis* can infect mammals, birds and possibly reptiles, although it is mainly observed in domestic livestock and other herbivores (Turnbull, 1996). The organism can be transmitted through carriage of spores from infected carcasses (e.g. by vultures or blow flies) to water or shrubs and trees. Bacilli can also be shed in the faeces of infected animals, and these sporulate on contact with air. Spores can also form in the tissues and exudates of animal carcasses. Once formed, spores may remain viable within the environment for decades (Turnbull, 1996). The most common reason for human infection is contact with dead or infected animals or contaminated products, or ingestion of infected meat. Infection occurs when spores enter the host and the mode of entry defines the type of infection. In the case of humans, there are three main forms of the disease: (a) cutaneous; (b) inhalational; and (c) gastrointestinal anthrax. Any of the types of infection can result in systemic anthrax, which is nearly always fatal (Mock and Fouet, 2001).



### **2.3.1 Cutaneous anthrax**

Cutaneous anthrax is the most common form of the disease in humans, accounting for more than 95% of reported cases. This occurs when the organism gains entry via a cut or abrasion in the skin (and the occasional report of transmission via an insect bite) followed by migration to the subepidermal tissue (Turell *et al.*, 1987; Bradaric *et al.*, 1992; Mallon and McKee 1997). The incubation period can vary from 9 hours to 2 weeks, but is typically 2-7 days. The infection initially presents as a papule which forms a ring of fluid filled vesicles around it and over a few days develops into a characteristic black necrotic lesion or 'eschar' accompanied by significant oedema. The skin lesions appear not to be suppurative as there is no abscess formation or tissue liquification. Histological examination of the lesions has, however, revealed lymphocytic infiltrates. Localised haemorrhaging, sometimes accompanied by thrombosis, may also be observed (Mallon and McKee 1997). Cutaneous anthrax is often self-limiting but can take up to six weeks to resolve, with or without appropriate treatment. Cutaneous anthrax usually remains localised to the site of infection and can be successfully treated with the use of suitable antibiotics (such as penicillin, deoxycillin or fluoroquinolones). Approximately 20% of untreated cases will result in systemic anthrax, in which clinical manifestations of disease progress to septicaemia, renal failure and death of the host (Hambleton and Turnbull, 1990).

### **2.3.2 Gastrointestinal anthrax**

Gastrointestinal anthrax occurs after ingestion of material contaminated with *B. anthracis*. There are two manifestations of the disease, intestinal or oropharyngeal. In the case of gastrointestinal anthrax it has been hypothesised that the bacterium infects the host at an opening in the mucosal lining (Dixon, 1999a, b). Histological examination shows the presence of vegetative bacilli in the submucosal and mucosal tissue although the exact location at which the spores germinate is unknown (Dutz, 1971). Significant mesenteric lymphadenitis is observed and ulceration of the stomach lining occurs, although whether this is localised at the point of infection or as a result of the action of the anthrax toxins is unclear (Dutz *et al.*, 1971, 1981). As in cutaneous anthrax, significant oedema and necrosis can be observed within infected tissue (Dutz *et al.*, 1981). Symptoms of intestinal anthrax include: fever; vomiting; abdominal pain, haematemesis; and bloody diarrhoea due to loss of blood, fluid and electrolyte imbalances (Ndyabahinduka *et al.*, 1984; Kanafani *et al.*, 2003). Blood may also be observed in vomit due to the extensive ulceration of the gut.

The rapid administration of effective treatment is essential to prevent systemic anthrax infection (Nalin *et al.*, 1977; Jena *et al.*, 1980; Ndyabahinduka *et al.*, 1984). However, in mild cases there is evidence to suggest that an individual can recover without treatment. Oropharyngeal anthrax has a mortality rate of approximately 50%, even with treatment. Clinical manifestations of this form of the disease include; a sore throat, local lymphadenopathy resulting in dysphagia, respiratory distress, and fever (Dogonay *et al.*, 1986). In this instance, lesions are observed in the oropharynx as pseudomembraneous ulcerations (Alizard *et al.*, 1995).

### **2.3.3 Inhalational anthrax**

Inhalational anthrax occurs when spores enter via the respiratory route, commonly from spores aerosolised from contaminated hides or other animal products. In a military context, inhalational anthrax is the most likely form of infection and one point of particular concern is that a biological hazard can exist at a significant distance from the point of dissemination (Manchee *et al.*, 1981; 1982; Sterne, 1982; Abranova *et al.*, 1993; Jackson *et al.*, 1998). *Bacillus* spores enter the host via the nasal passages and are subsequently deposited in the lungs; however, in most cases infection at this site has not been observed. Bacterial spores are phagocytosed by host macrophages at the primary site of infection and are transported to the mediastinal and peribronchal lymph nodes. Germination of the spores occurs as the macrophages migrate to these lymph nodes, within which outgrowth occurs causing mediastinitis and subsequent spread through the lymphatic system and bloodstream. Early diagnosis of inhalational anthrax can be particularly problematic as an infected individual will often present with mild non-specific symptoms such as malaise or fever. However, this is followed by the rapid onset of severe symptoms characteristic of the systemic form of the disease such as dyspnoea, cyanosis and disorientation. The severity of infection is usually greatest in the case of inhalational anthrax (Webb, 2003). Death results within 2-3 days due to shock, sepsis and respiratory failure (Turnbull, 1991). Systemic inhalational anthrax has a mortality rate of close to 100% and the LD<sub>50</sub>, based on studies using primates could be in the region of  $2.5 \times 10^3$  to  $5.5 \times 10^4$  inhaled spores for humans (Inglesby *et al.*, 2002). The timing of the administration of effective treatment is critical to survival. Oral antibiotics, if taken during the presymptomatic incubation period, can be very effective. In the presymptomatic stage, combination antibiotics and intensive hospital care can sometimes be effective (Lincoln *et al.*, 1965; Inglesby *et al.*, 2002). However, once the disease has reached the fulminant stage and a critical threshold has been reached with regard to the production of the anthrax toxins, antibiotic therapies become ineffective (Brachman, 1980). Natural isolates of *B. anthracis* are susceptible to a range of antibiotics

such as penicillin, doxycycline or ciprofloxacin (Jones *et al.*, 2003a). However, in a BW scenario, these treatments may be ineffective if a strain has been engineered to allow for resistance to a range of commonly used antibiotic therapies. Work conducted by Stepanov *et al.* (1996) detailed the genetic modification of a vaccine strain of *B. anthracis* to confer resistance to penicillins and tetracyclines.

### 2.3.4 Anthrax meningitis

Anthrax meningitis develops as a complication of other forms of the disease, with a mortality rate approaching 100% (Dixon *et al.*, 1999b). Only a few cases have been described of recovery following rapid diagnosis and treatment (Khanne *et al.*, 1989; Lalitha *et al.*, 1996). Patients may present with symptoms often observed with a meningeal infection and also fever, myalgia, vomiting, seizures and delirium. Clinical symptoms include marked inflammation of the meninges, elevated cerebrospinal fluid pressure and appearance of blood in this fluid due to haemorrhaging, followed rapidly by death. An autopsy will also reveal the presence of the characteristic 'cardinal's cap', due to haemorrhaging of the leptomeninges (Koshi *et al.*, 1981; Levy *et al.*, 1981; Abranova *et al.*, 1993; George *et al.*, 1994; Lalitha *et al.*, 1996).

### 2.4 Virulence factors

The major virulence factors are encoded on two plasmids, pXO1 and pXO2; namely two toxins involved in cell death and evasion of the host's immune response and the poly- $\gamma$ -D-glutamic acid capsule, which inhibits the phagocytosis of vegetative bacilli and dissemination of the organism from the lungs (Leppla, 1991; Drysdale *et al.*, 2005). It has been hypothesised that the toxins are predominantly responsible for the morbidity and mortality of anthrax infection and the capsule for evasion of the host's immune system and thus survival *in vivo* (Hanna, *et al.*, 1998; Mock and Fouet, 2001; Ascenzi *et al.*, 2002.). Expression of the major virulence factors is affected by host-specific factors including carbon dioxide (CO<sub>2</sub>) concentration ( $\geq 5\%$ ), elevated host temperature ( $\geq 37\%$ ) and the presence of serum components (Makino *et al.*, 1988; Dai *et al.*, 1995). The expression of both toxin and capsule genes is controlled by the transcriptional activator *atxX*. Loss of either or both plasmids results in reduction in virulence. Attenuated strains have been used

as vaccines, such as the Sterne stain (pXO1<sup>+</sup>pXO2<sup>-</sup>) which produces the toxins but is unable to synthesise the capsule.

#### 2.4.1 pXO1

The pXO1 plasmid is a 184.5 kilobase pair (kbp) element, that encodes three toxin genes along with other regulatory genes, a resolvase, a transposase and a germination operon, termed *gerX* (Okinaka *et al.*, 1999a; Mock and Fouet, 2001). On the plasmid is a pathogenicity island within which the major virulence genes are located within a 44.8-kbp region which is flanked by inverted IS1627 elements. Genes also encoded within pXO1 include DNA topoisomerase (Fouet *et al.*, 1994) and 15 Open Reading Frames (ORFs) which have been implicated in horizontal transfer (Mock and Fouet, 2001).

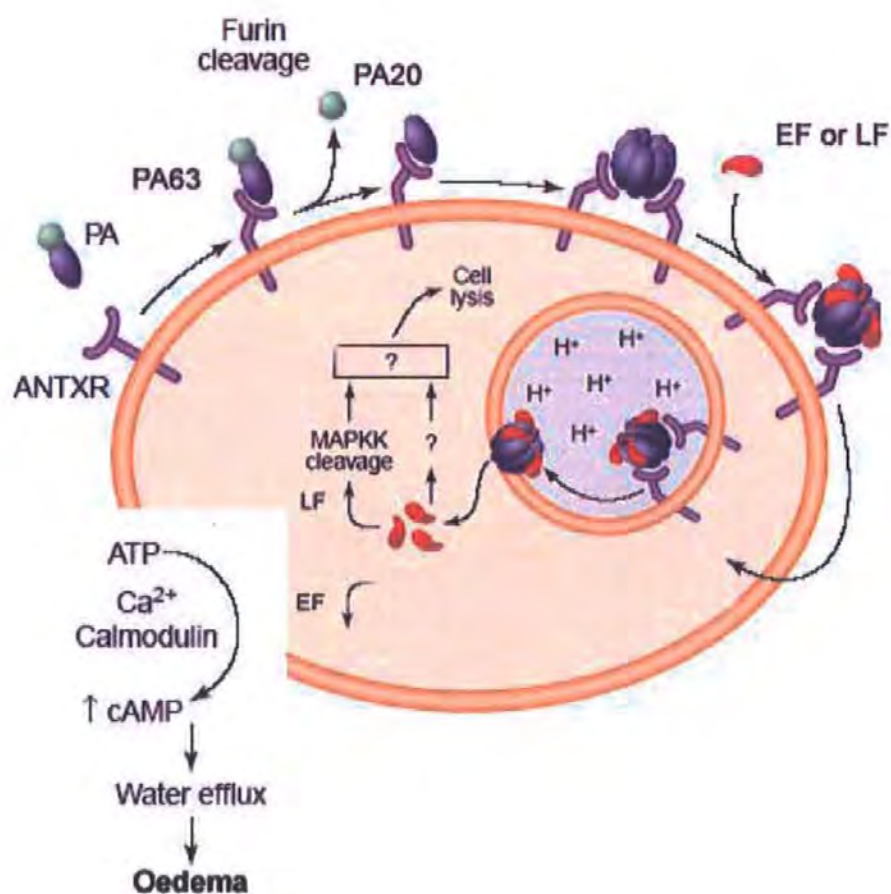
The *atxA* gene is also located on the pXO1 plasmid within the pathogenicity island and is flanked by *pagA* and *cyaA*. The *atxA* gene encodes a 476 amino acid (aa) protein that exerts positive and negative control over genes located on pXO1, pXO2 and chromosomal elements (Vodkin, and Leppla, 1983; Mock *et al.*, 1988; Robertson, *et al.*, 1988; Tippetts and Robertson, 1988; Welkos *et al.*, 1988; Uchida, *et al.*, 1993a; Koehler *et al.*, 1994; Dai *et al.*, 1995; Guignot *et al.*, 1997; Hoffmaster and Koehler, 1997; Bragg *et al.*, 1999; Bourgogne *et al.*, 2003; Mignot *et al.*, 2003). Evidence suggests that *atxA* is affected by a number of host factors such as temperature and CO<sub>2</sub> (Dai and Koehlar, 1997; Uchida *et al.*, 1997, Mock and Mignot, 2003). A tricistronic operon named *gerX* is also located on pXO1. Deletion of this operon has found to have an effect on the virulence *in vivo* using a murine model (Guidi-Rontani *et al.*, 1999a). The *gerX* operon is thought to encode proteins that have a role to play in the germination of *B. anthracis* within the aveolar macrophage. However, conflicting results demonstrated the presence of this operon within an attenuated vaccine strain 'Carbosap' (Adone *et al.*, 2002). Investigation of this strain also revealed the presence of other common virulence determinants such as the toxin encoding genes and the two trans-acting regulators *acpA* and *atxA*. This suggests a more complex mechanism for the regulation and expression of virulence factors than has been elucidated to date and also highlights the care that is required for identification of pathogenic *B. anthracis* strains based on genetic methods (discussed in more detail in section 2.8.1).

## 2.4.2 Anthrax toxins

The structure and function of each component of the two *B. anthracis* toxins have been characterised and reviewed extensively (Escuyer *et al.*, 1988; Robertson *et al.*, 1988; Welkos *et al.*, 1988; Bragg and Robertson, 1989; Ascenzi *et al.*, 2002;; Brossier and Mock, 2001; Mourez *et al.*, 2002; Collier and Young, 2003). Anthrax toxins consist of three subunits; oedema factor (EF), lethal factor (LF) and protective antigen (PA) encoded by the genes, *cya*, *lef* and *pag* respectively which are all located on the pXO1 plasmid (Dai *et al.*, 1995; Hoffmaster *et al.*, 1997; Okinaka *et al.*, 1999; Mock and Fouet, 2001). The individual subunits are non-toxic, but combine to form two binary toxins LF-PA (Lethal toxin or LeTx) and EF-PA (oedema toxin or EdTx). The binary toxin is of the A-B toxin family, the A moiety (LF or EF) acting on targets within the cytosol, and the B moiety binding to a specific host cell receptor and allowing translocation of A into the cytosol (Ascenzi *et al.*, 2002). Each of the toxins has a specific role in the pathogenicity of *B. anthracis*. Guidi-Rontani (2002) hypothesised that the toxins disrupted cells to allow the bacteria to enter the bloodstream.

An overview of toxin entry and action is shown in Figure 2.1. Protective antigen (PA) binds to a surface located host receptor. To date, two anthrax receptors have been identified; these are tumour endothelial marker (TEM8 or ANTX1) (Bradley *et al.*, 2001) and capillary morphogenesis protein 2 (CMG2 or ANTX2) (Scobie *et al.*, 2003). Cleavage of PA occurs by a furin-like cell-surface membrane protease, releasing a PA<sub>20</sub> subunit. The C-terminal 63 kDa protein subunit (PA<sub>63</sub>) remains bound to the host cell and oligomerizes to form [PA<sub>63</sub>]<sub>7</sub>, a heptameric ring, capable of binding a maximum of three molecules of LF or EF. Recent work conducted by Christensen *et al.* (2005) suggests that there is a rapid phase of dissociation of PA<sub>20</sub> from PA<sub>63</sub> followed by a slow isomerisation phase which has been hypothesised to allow for oligomerisation of PA<sub>63</sub> in the presence of LF and EF. The resulting toxin complex is internalised through clathrin mediated endocytosis (Ascenzi *et al.*, 2002) and transported to an acidic endosome. Evidence suggests that membrane insertion and translocation of LF and EF across the membrane and into the cytosol is promoted through a conformational change in the PA oligomer, triggered by the acidic pH in the endosomal compartment (Larantz *et al.*, 2004). Simultaneous initiation of these events, triggered by low pH, would appear to be essential, as, if a pore were formed before translocation could occur, there may be a change in membrane potential and permeability, leading to death of the host cell (van der Groot, 2001). If cell death was triggered, then this in turn could initiate an inflammatory response. Evasion of this response is thought to be critical to anthrax pathogenesis (Park *et al.*, 2002; Agrawal *et al.*, 2003; Moayeri and

Leppä, 2004; Fukao *et al.*, 2004). PA also appears to insert preferentially into intraluminal vesicles rather than into endosomal carrier vesicles (ECV) or multivesicular bodies (MVB) and these are finally translocated along microtubules and into late endosomes. This mechanism ensures that after translocation, LF and/or EF are present within the lumen and not the cytoplasm of the endosome; thus they can refold, and are protected from lysosomal protease degradation (Abrami *et al.*, 2004). Release of EF and LF has been hypothesised to be due to a back fusion event whereby intraluminal vesicles fuse with the limiting membrane during the late stages of the endocytotic pathway (Abrami *et al.*, 2004). Once released into the cytoplasm, EF and LF are then able to bind to their target molecules.



**Figure 2.1** Mode of action of *B. anthracis* toxins (Adapted from Sánchez and Bradley, 2004).

#### **2.4.2.1 Protective antigen (PA)**

PA is an 83 kDa protein that is essential for host cell recognition, binding and subsequent translocation of LF or EF from the extracellular space to the cytoplasm. PA has four domains; one domain is responsible for binding to the anthrax toxin receptor (ATR), a type I membrane protein (Bradley *et al.*, 2001). The second domain of PA contains two calcium ions and a site allowing for cleavage by a furin type protease. The third domain of PA is involved in oligomerisation to form a heptameric ring structure which can bind to LF and/or EF at a 252 aa region of the N-terminus of PA (Singh *et al.*, 1994; Mogridge *et al.*, 2001; Cunningham *et al.*, 2002; Lacy *et al.*, 2002). The fourth domain is a large flexible loop that has been hypothesised to interact with the host receptor and has a role in pore formation (Santelli *et al.*, 2004; Rainey *et al.*, 2005).

#### **2.4.2.2 Oedema toxin (EdTx)**

EdTx forms as a result of binding of EF to PA. EF is an 89 kDa calmodulin or calcium dependent adenylate cyclase that causes a dramatic increase in cyclic AMP (cAMP) levels within the host cell, resulting in an imbalance of water homeostasis, thought to cause the significant oedema observed with cutaneous anthrax (Leppla, 1982). Initial investigations of the effects of EdTx using a skin test on animal models resulted in only localised oedematous necrotic lesions (Thorne *et al.*, 1960; Stanley *et al.*, 1961). *In vitro* testing also demonstrated the ability of EdTx to inhibit neutrophil function, an effect that has also been observed in human cases of cutaneous anthrax (O'Brien *et al.*, 1985; Alexeyev *et al.*, 1994). However, more recent investigation using highly purified EdTx suggest that it causes a series of rapid pathological effects, finally resulting in death of the host, in a murine model (Firoved *et al.*, 2005).

#### **2.4.2.3 Lethal toxin (LfTx)**

Lethal toxin is formed through binding of LF to PA. LF is a Zn<sup>2+</sup> metalloprotease which affects the mitogen activated protein kinase signalling pathway through cleavage of host mitogen activated protein kinase kinases (MAPKKs or MEKs) with the exception of MEK5 (Duesbery *et al.*, 1998; Vitale *et al.*, 1998; Vitale *et al.*, 2000). Evidence suggests that LfTx can exert a multitude of effects on a wide range of cells involved in the immune response (Park *et al.*, 2002; Fukao *et al.*, 2004; Moayeri and Leppla, 2004). For example, it has been demonstrated that anthrax LfTx can inhibit cytokine production and induce the release of immunomodulatory molecules such as tumour necrosis factor (TNF- $\alpha$ ) and nitric

oxide (NO) by lipopolysaccharide (LPS) or  $\gamma$ -interferon (Pelizzari *et al.*, 1999; Erwin *et al.*, 2001). It has also been shown that LfTx lyses macrophages in certain murine models and can trigger death of human macrophages when sensitised by LPS or TNF (Chaudry *et al.*, 2002; Mourez *et al.*, 2002; Moayeri *et al.*, 2003, 2004). Agrawal *et al.* (2003) demonstrated that anthrax LfTx also has an effect on adaptive immunity using mouse and human dendritic cells. After exposure to LfTx and subsequent exposure to LPS, a significant reduction of secreted proinflammatory cytokines (e.g. TNF- $\alpha$ , IL-1 $\alpha$ , IL-6 and IL-12) was observed, coupled with no detectable up-regulation of co-stimulatory molecules.

### 2.4.3 *pXO2*

The smaller *pXO2* plasmid (95.3 kbp) contains 85 predicted ORFs and encodes the genes required for the capsule biosynthetic operon (*capBCADE*) (Uchida, *et al.*, 1993b, Makino *et al.*, 1988, 1989, 2002), degradation and regulatory elements. Transposon mutagenesis studies conducted by Welkos *et al.* (1991) demonstrated that mutations within some *pXO2* genes may allow for synthesis of the capsule at levels observed in the wildtype. However, a reduction in the LD<sub>50</sub> was often observed using a murine subcutaneous model of infection. This strongly suggests that other, as yet unidentified genes encoded within *pXO2* may also have a role to play in virulence. Transcription of the *cap* genes occurs as a single operon and the individual genes encode the proteins required for synthesis, assembly and attachment of the capsule to the cell surface (Makino *et al.*, 1988, 2002). The expression of toxin and capsule genes is regulated by a transcriptional activator *AtxA*, encoded within *pXO1* (Uchida *et al.*, 1993a; Koehler *et al.*, 1994; Dai *et al.*, 1995; Bourgogne *et al.*, 2003). Recent work has shown that genetic regulation of the capsule genes is more complex than originally proposed (Drysdale *et al.*, 2004, 2005). As well as *atxA* the regulation of capsule gene expression is also governed by two *pXO2* located transcriptional activators; *acpA* (Vietri *et al.*, 1995) and more recently, a second regulatory element, *acpB*, has been identified (Drysdale *et al.*, 2004). Both *AcpA* and *AcpB* are homologous to each other (~62% identity) and to *AtxA* (~50% identity). The *atxA* gene is suggested to control the expression of the capsule genes through the positive regulation of *acpA* and *acpB* (Drysdale *et al.*, 2004); however, this transcriptional regulation appears to be unidirectional as *acpA* was observed to have no effect on the expression of the toxin genes (Bourgogne *et al.*, 2003; Fouet and Mock, 1996; Uchida *et al.*, 1997). *AcpA* and *AcpB* also appear to have partial functional overlap (Drysdale *et al.*, 2004). Originally *acpB* was thought to have a minor role in the regulation of capsule gene expression



(Drysdale *et al.*, 2004). However, evidence from more recent research conducted by the same group (Drysdale *et al.*, 2005), found that an *acpB* but not an *acpA* mutant showed increased LD<sub>50</sub> values in a murine model. This suggests a more predominant role for this regulatory element in the regulation of other genes required for virulence.

#### 2.4.4 The capsule

Capsules are observed in many pathogenic bacteria and are required for virulence through evasion of the host's immune response (Cunnion *et al.*, 2001; Dale *et al.*, 1996; Domenico *et al.*, 1994). Unlike the polysaccharide capsules observed in a range of pathogenic bacteria, *B. anthracis* synthesises a highly negatively charged  $\gamma$ -linked D-glutamic acid ( $\gamma$ PGA) which forms long homopolymers, often observed at greater than 215 kDa, which make up the capsule (Record and Wallis, 1955; Zwartou and Smith, 1956). *In vitro* capsule synthesis occurs in bicarbonate rich media, or when the organism is grown under conditions of 5 to 20% CO<sub>2</sub> (Meynell and Meynell, 1964; Green *et al.*, 1985; Thorne, 1993). The capsule has an important role in *B. anthracis* pathogenesis, as non-encapsulated mutants are significantly attenuated (Ivins *et al.*, 1986; Drysdale *et al.*, 2005). The effect of the presence of the capsule on *B. anthracis* pathogenesis is two fold. Firstly, it decreases the degree of binding to complement components thus inhibiting phagocytosis (Mock and Fouet, 2001). Secondly, its simple composition renders it non- or weakly immunogenic thus, a significant reduction or elimination of any form of immune response is observed (Leppla, 1991; Keppie *et al.*, 1953; Leonard and Thorne, 1961; Makino *et al.*, 1989; Schneerson *et al.*, 2003). There are five different Cap proteins expressed by transcription of the *capBCADE* operon (CapB, CapC, CapA CapE and CapD). CapD is a 51 kDa enzyme that releases lower molecular weight glutamic acid polymers into the external environment through depolymerisation of larger D-glutamic acid polymer chains. These degraded polyglutamates are predicted to distract or inhibit the host defence mechanisms, allowing infection to become established (Uchida *et al.*, 1993b) and appear to be essential for vegetative cell outgrowth *in vivo* (Makino *et al.*, 2002). The CapE protein has been identified more recently and is a 47 aa peptide located within the membrane of *B. anthracis* and is also required for polyglutamate synthesis and appears to interact with CapA (Candela *et al.*, 2005). A number of groups have recently shown the ability of glutamic acid polymers to elicit an immune response when conjugated to a carrier (Rhie *et al.*, 2003; Schneerson, *et al.*, 2003; Wang *et al.*, 2004). Thus, there is renewed interest in the utilisation of the capsule as a marker for detection and diagnosis and in the development of an effective treatment or prophylaxis for anthrax infection (Chabot *et al.* 2004).

#### 2.4.5 Other virulence determinants

To date much of the research on virulence determinants in *B. anthracis* has focused on the action of the anthrax toxins and to a lesser extent, the capsule; however, more recently research has begun on the examination of other *B. anthracis* virulence factors. Work conducted by Xu *et al.* (2004) identified two adhesins, BA0871 and BA5258, which are homologues of a collagen binding protein found within *Staphylococcus aureus*. Binding of the recombinant form *B. anthracis* adhesins to collagen was demonstrated by two different methods. The first of these was Surface Plasmon Resonance (SPR), where the binding of one molecule to another can be monitored due to a change in the angle of incidence at which a proportion of light energy can interact with delocalised electrons (plasmons) formed in a metal film, commonly gold. The second method used was the more commonly known enzyme linked immunosorbant assay (ELISA). Xu *et al.* (2004) also observed the involvement of these proteins in mediating cell attachment to collagen. Thus, both proteins were hypothesised to be involved in establishing anthrax infection, and possibly of greatest significance in the cutaneous form of the disease (Xu *et al.*, 2004). More recently, the role of rhamnose in *B. anthracis* pathogenesis was also investigated (Bozue *et al.*, 2005). There are two isomers of rhamnose, D- and L-rhamnose. The most common isomer is L-rhamnose which is found in a variety of Gram positive and Gram negative bacteria and functions as a structural component of the cell wall or capsule (Giraud and Naismith, 2000). The enzymes required for rhamnose biosynthesis are encoded within an *rmlABCD* operon. After examination of mutations within the *rmlABCD* operon it was reported that rhamnose was important for interaction with macrophages *in vitro*, but did not affect virulence in a guinea pig model where the spores were administered intramuscularly (Bozue *et al.*, 2005). This mirrors observations conducted on the virulence of other exosporial components in animal models (discussed in section 2.5.2.1), where mutations in these genes have no observed affect on virulence; however, the application of an inhalational model may be more relevant (Kang *et al.*, 2005). A number of enzymes, in particular proteases, have also been put forward as having a role in *B. anthracis* virulence. However, although, *B. anthracis* contains full structural genes for a number of pathogenic factors, such as lysolecithinases, hemolysins or phospholipases it only secretes a small proportion of these, believed to be due to a missense mutation in the *plcR* gene. Pophov *et al.* (2005) examined the potential role of particular proteolytic enzymes in the pathogenicity of *B. anthracis*. In this case, examination of chromosomally encoded genes identified a number of putative virulence factors, including collagenases, haemolysins,

phospholipases and other toxins. Experimental evaluation was conducted using an Ames derivative cured of both pXO1 and pXO2, and thus unable to synthesise the toxins or capsule. Aronson *et al.* (2005) demonstrated the regulation of chromosomal proteases, implicated with a role in virulence, by pXO1 encoded regulatory elements, as has also been observed with the regulation of the S-layer proteins Extractable Antigen 1 (EA1) and the Surface array protein (Sap) by *atxA* (Mignot *et al.*, 2003). Whether all of the genes regulated by *atxA* have a role in pathogenesis of *B. anthracis* has yet to be fully elucidated. This research demonstrated the complexity of the acquisition of the highly pathogenic and virulent phenotype observed in *B. anthracis* and may suggest that the key to genetic discrimination between virulent, attenuated and avirulent strains may lie within regulatory regions.

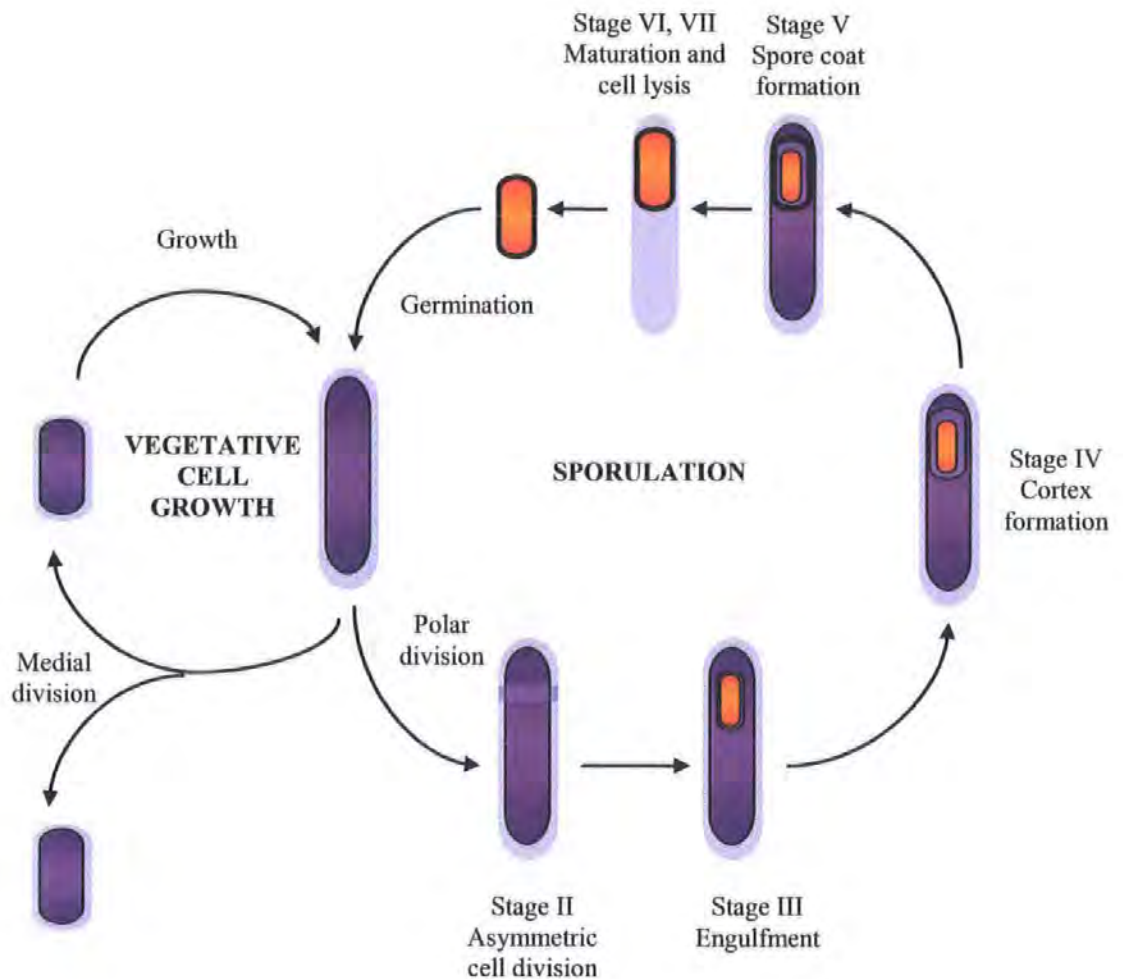
## **2.5 The *Bacillus anthracis* spore**

As the spore is the infective form of *B. anthracis* (Dixon *et al.*, 1999b; Liu *et al.*, 2004), understanding its structure is essential in the identification of detection targets. The knowledge gained from the identification of outer surface targets could also be used in preventive therapies and early treatment of an anthrax infection.

### **2.5.1 Sporulation**

From extensive work, conducted predominantly using *B. subtilis*, the process of sporulation and its genetic control has been extensively characterised and described in a number of recent reviews (Henriques and Moran, 2000; Sonenshein, 2000; Kroos and Yu, 2000; Stephenson and Hoch, 2002; Piggot and Losick, 2002; Errington, 2003; Ryan and Shapiro, 2003; Hillbert and Piggot, 2004). The following is a brief overview of this process, and covers a number of important genes and their products but does not discuss all those involved in the sporulation cycle as this is beyond the scope of this study.

Spores usually form as the vegetative cells pass out of the exponential growth phase or in response to an environmental signal that will limit cell growth, typically nutrient depletion (Straiger and Losick, 1996). In *B. subtilis*, sporulation has been assigned seven phases from morphological examination by electron microscopy (Ryter, 1967). Each transitional phase of sporulation is completed over a period of approximately one hour and occurs as a continuous cycle (Figure 2.2).



**Figure 2.2** A diagrammatic representation of seven key stages of sporulation, adapted from Errington (2003).

Stage 0 is defined as the vegetative cell once committed to sporulation. Stage I is characterised by the condensation of two replicated chromosomes, which form an axial filament due to alignment along the longitudinal axis of the cell. As the vegetative cell enters Stage II, asymmetric cell division, a process similar to medial cell division observed in the vegetative cell state, occurs. At this stage, a septum forms dividing the cell (sporangium) to form the prespore and the mother cell, each containing a single chromosome. At stage III, hydrolysis of the peptidoglycan occurs, followed by the engulfment of the prespore by the septal membranes to form the forespore. The free protoplast is thus bounded from the cytoplasm of the mother cell by the inner and outer forespore membranes. At stage IV, the peptidoglycan of the cortex then forms around the forespore, followed by the cortex. These structures are assembled between the membranes surrounding the forespore. This is followed by assembly of the spore coat proteins,

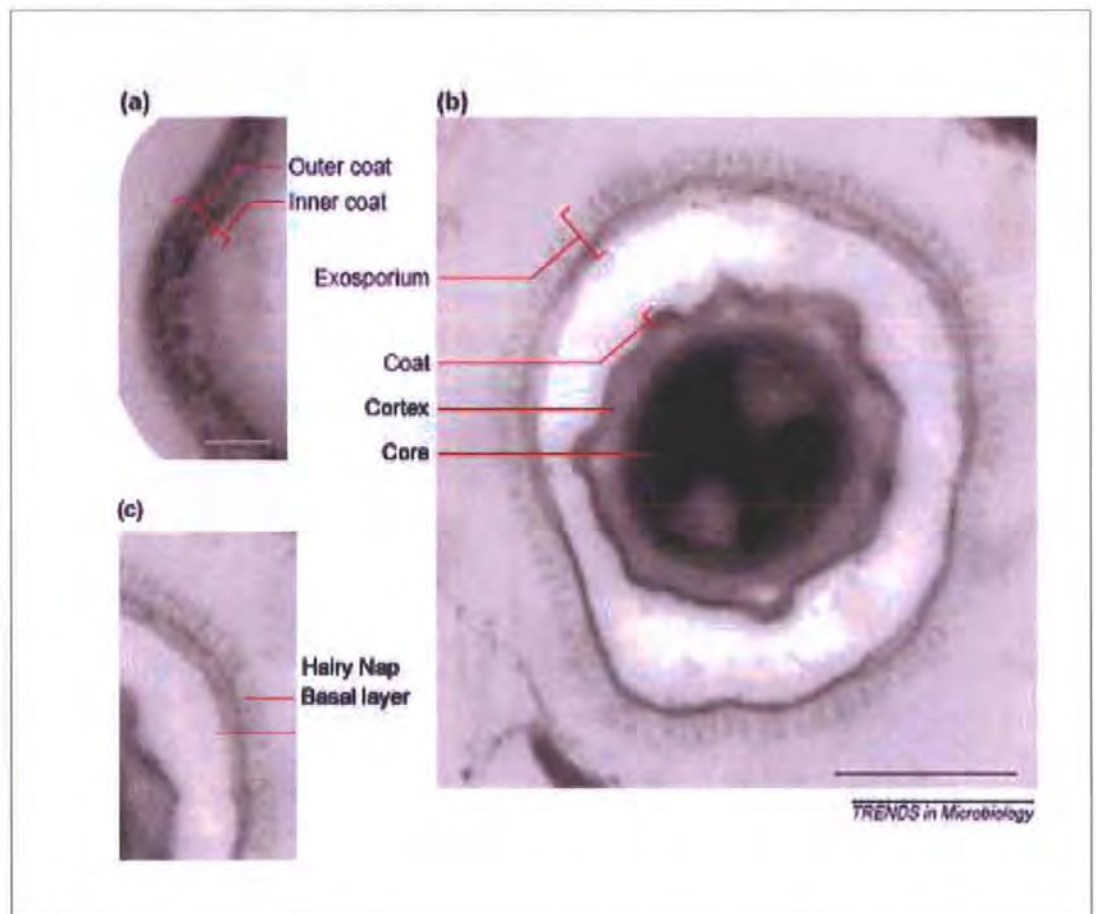
synthesised by the mother cell, on the outer membrane (Stage V). At Stage VI spore maturation occurs and the spore acquires the characteristic properties of resistance, dormancy and the ability to germinate. There is no significant observable morphological change to the spore during this phase. The final step in sporulation (Stage VII) is lysis of the mother cell to release a fully mature spore (Henriques and Moran, 2001; Driks, 1999).

Under favourable environmental conditions, the spore germinates. The spore interior is rehydrated, the spore coat hydrolyses, the cortex is shed, metabolism is activated and vegetative cell growth is resumed (Piggot and Coote, 1976; Foster and Johnstone, 1990; Setlow, 2003).

Initiation and regulation of sporulation is controlled by the expression of a number of genes in the forespore and the mother cell (Driks, 1999). In the case of *B. subtilis*, entry of the bacterium from the vegetative to the dormant state is directed by a temporally and spatially separated cascade of proteins termed sigma factors (Moran *et al.*, 1981; Strauch *et al.*, 1990; Weir *et al.*, 1991; Margolis *et al.*, 1993; Resnekov *et al.*, 1995). The first sigma factor activity to increase is  $\sigma^H$ , which along with  $\sigma^A$  effects the expression of a number of genes involved in sporulation. Once the septum has formed,  $\sigma^E$  and  $\sigma^F$  become active in the mother cell and forespore compartment, respectively (Margolis *et al.*, 1991). After engulfment of the forespore,  $\sigma^E$  and  $\sigma^F$  are replaced by  $\sigma^K$  and  $\sigma^G$ , respectively.  $\sigma^K$  also activates a number of genes involved in late sporulation, most notably synthesis of the cortex and the majority of the coat proteins (Margolis *et al.*, 1991). Finally, GerE and  $\sigma^K$  both coordinate the last phase of gene expression within the mother cell (Cutting *et al.*, 1991; Roel and Losick, 1995). A more detailed review of the genetic control of spore formation in *B. subtilis* has been described by Driks (1999) and Errington (2003). From comparison of the *B. subtilis* and *B. anthracis* genome it was observed that important structural and regulatory genes involved in sporulation are present and show >50% identity (Aronson, 2002). Other well conserved regions in the *B. anthracis* genome include genes encoding SASPs and a number of coat proteins. Examination of the *B. anthracis* genome suggests that many of these genes are similar, if not identical, to those observed in *B. subtilis* (Aronson, 2002). The presence of many of the same genes or gene homologues suggests that many of the mechanisms of sporulation are similar (Aronson, 2002).

### 2.5.2 Spore structure

During the dormant phase, spores are characterised as phase bright and ellipsoidal using phase contrast microscopy (Piggot and Coote, 1976; Nicolson and Setlow, 1990). The highly refractive nature of a bacterial endospore is dependent on the degree of dehydration, which in turn is reliant on correct assembly of the cortex. In the dormant state, spores are cryptobiotic and possess a highly ordered structure conferring resistance to extreme environmental conditions such as heat, ionising radiation, ultra-violet (UV) radiation, pressure and chemicals (Setlow, 1994; Nicholson *et al.*, 2000; Takamatsu, 2002). The basic structure of *B. anthracis* spores is shown in Figure 2.3.



**Figure 2.3** The basic structure of *Bacillus* spores by Transmission Electron Microscopy. (a) The inner and outer coat layers of a *B. subtilis* spore, scale bar 100nm (b) A cross section of a *B. anthracis* spore showing the exosporium, the thinner inner coat, cortex, outer coat and the core. (c) The hairy nap and basal layers of the *B. anthracis* exosporium. Scale bar 300 nm (*B. anthracis*). Adapted from Driks (2002).



### 2.5.2.1 *The exosporium*

In the case of *B. anthracis* and other members of the *B. cereus* group, the surface of the outer spore coat has been found to be covered by a loose balloon like structure termed the exosporium (Ohye and Murrell, 1973; DesRosier and Lara, 1984; Driks, 1999). This surrounds, but is not attached to, the other spore components (Gerhardt, 1967; DesRosier and Lara, 1984). The exosporium forms the outermost integument between the spore and its external environment (Gerhardt, 1967). The exosporium was thought to be absent in some *Bacillus* species, such as *B. subtilis*. However, recent research using ruthenium red staining has demonstrated the presence of a thin glycoprotein layer surrounding the spores of this species and as such this has been proposed to be a previously unidentified thin exosporial outer surface layer (Waller *et al.*, 2004). Typically, the exosporium consists of many layers that surround the spore itself (Gerhardt; 1967; DesRosier and Lara, 1984). Analysis of the exosporium revealed that it consisted of a paracrystalline basal layer and a “hair like” outer layer, also termed the hairy nap (Roth and Williams, 1963; Hachisuka *et al.*, 1966). The crystalline nature of the basal layer has also been observed to be conserved on both inner and outer sides (Sylvestre *et al.*, 2002). The exosporium also displayed a close-packed hexagonal lattice structure (Gerhardt and Ribi, 1964).

The exosporium itself is composed of a number of proteins, of which a proportion have been identified (Steichen *et al.*, 2003; Todd *et al.*, 2003; Lui *et al.*, 2004; Redmond *et al.*, 2004). However, the function of the majority of these proteins is as yet undetermined. The first structural exosporium protein to be identified was the glycoprotein BclA (Sylvestre *et al.*, 2002). Since its identification, a number of studies have been conducted to characterise this protein. BclA was found to be the primary structural component of the hair like filaments and reported to be similar to collagen due to the presence of a number of centrally located GXX (usually GPT) repeats. Sylvestre *et al.* (2002) demonstrated that, although the protein has a clear structural role within the exosporium, it is not essential for virulence in a subcutaneous model of infection in mice, and does not contribute to the resistance of spores to harsh chemical treatment. However, investigation of the role of BclA in an inhalational model has not been determined, where BclA may have a specific function with regards to this route of infection such as deposition and adhesion to the lung cells. Sylvestre *et al.* (2003) not only demonstrated the presence of BclA in a wide range of *B. anthracis* isolates but showed considerable size heterogeneity among different *Bacillus* species and strains. The protein has different numbers of GPT repeats and [GPT]<sub>5</sub>GDTGTT repeats and it has been hypothesised that these variations could play a

role in the properties of the spores in different environments (Sylvestre *et al.*, 2003). The protein consists of three domains; a 19 aa N-terminal domain, a central region containing the collagen-like repeats and a C-terminal domain of 134 aa (Sylvestre *et al.*, 2002; Steichen *et al.*, 2003). Analysis of the orientation of these filaments by electron microscopy suggested that the C-terminal domain is located at the distal end and the N-terminus is located at the proximal end of each hair like filament, suggesting a role for the N-terminal region in binding to the basal layer. BclA was also shown to form a collagen like triple helix. However, of note was the observation that these trimers demonstrated a significant increase in thermal stability ( $T_m$  95°C), compared to mammalian collagen ( $T_m$  37°C). This was also observed in trimers formed only by the C-terminal domain. Furthermore, these trimers were also found to be resistant to degradation by a number of proteases, high temperatures and concentrations of detergent, and when purified the formation of large crystalline sheets was observed (Boydston *et al.*, 2005). These results suggest that BclA may form a protective barrier around the spore; however, given the resistance of spores devoid of an exosporium to harsh environmental conditions, the likelihood is that this protective barrier has a more important function in the interaction of the spore with the host (Kang *et al.*, 2005). Daubenspeck *et al.* (2004) identified novel oligosaccharide side chains of BclA; a 715 Da tetrasaccharide, of which multiple copies are present within the collagen like region of the protein and a 324 Da disaccharide that may be attached outside of this region. The 715 Da tetrasaccharide was found to be composed of 3 rhamnose residues and a component termed anthrose. The 324 Da disaccharide was composed of a single rhamnose residue (3-*O*-methyl-rhamnose). Daubenspeck *et al.* (2004) also hypothesised that the anchor points for rhamnose oligosaccharides within BclA were the characteristic GPT and [GPT]<sub>5</sub> repeats. The *bclA* gene is encoded in a cluster composed of 11 ORFs which are also hypothesised to contain genes involved in carbohydrate synthesis, more specifically for the spore specific carbohydrate 3-*O*-methyl rhamnose, also associated with BclA, flanked by *spoVE* homologue and enoyl-acyl carrier protein reductase. This was inferred as homologous genes were present in the thermophilic bacterium *Aneurinibacillus thermoaerophilus* in which these genes have a role in the synthesis of rhamnose and 3-*O*-methyl rhamnose (Daubenspeck *et al.*, 2004).

Steichen *et al.* (2003) demonstrated that BclA was an immunodominant protein of the *B. anthracis* exosporium, during their evaluation of a number of monoclonal antibodies raised against  $\gamma$ -irradiated spores. Their study also revealed the presence of alanine racemase (



molecular weight. ca. 43 622 Da), iron/manganese superoxide dismutase, SOD (ca m.w. 24 600 Da) and two other proteins termed BxpA (m.w. ca. 12 842) and BxpB (ExsF/ExsFA) (m.w. ca. 17 331 Da). All these proteins have homologues within *B. cereus*. The nature of the association of alanine racemase and SOD with the spore surface is at present unclear. They may perform essential roles and/or be tightly adsorbed to the exosporium. Alanine racemase is an enzyme which can convert the germinant L-alanine to the germination inhibitor D-alanine and so has a role in the regulation of germination. It has been suggested that the alanine racemase (along with the inosine hydrolase also isolated within the exosporium) may suppress germination through degradation of L-alanine or inosine, respectively (Gould, 1966; Titball and Manchee, 1987; Steichen *et al.*, 2003; Redmond *et al.*, 2004). SOD has been hypothesised to have a number of possible functions within the spore, such as involvement in the oxidative cross-linking of exosporium and/or spore coat proteins (Henriques and Moran, 2000) or protection from reactive oxygen species (Lynch *et al.*, 2000). Spores are highly resistant oxidative stress but it has not been determined if this property is due to the ability of the spore to scavenge reactive oxygen species (Inaoka *et al.*, 1999). However, the superoxide dismutase SodA of *Bacillus subtilis* has been shown to be essential for protection against oxidative stress in sporulating cells and is required for maturation of the spore (Henriques *et al.*, 1998; Inaoka *et al.*, 1999). More recently, it has been suggested that SOD may regulate the germination of *B. anthracis* spores on exposure to superoxide (Baillie *et al.*, 2005). It was suggested that BxpA and BxpB (ExsF/ExsFA) may both have a role in the structure of the *B. anthracis* exosporium and that BxpB (ExsF/ExsFA) was glycosylated or associated with glycosylated material. The latter seems the most likely as ExsF forms a complex with BclA (Redmond *et al.*, 2004). Sylvestre *et al.* (2005) indicated that the ExsFA (ExsF/BxpA) and a second protein ExsFB are required for the correct localisation of BclA to the spore surface and for the stability of the paracrystalline basal layers of the exosporium. (Sylvestre *et al.*, 2005). Later work by Steichen *et al.* (2005) also suggested a role for BxpB, (ExsF/ExsFA) in the suppression of germination, although no effects on the spores resistance was observed.

Fujita *et al.* (1989) demonstrated that, in the case of *B. subtilis*, some vegetative cell membrane proteins are also present in the spore coat indicating that some of the outer forespore membrane is conserved within the dormant spore. Vegetative cell proteins are also found within the *B. anthracis* spore (Steichen *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004). This observation has raised uncertainties as to whether some proteins within exosporium preparations are true structural components or alternatively strongly adsorbed.

Thus, some studies have included salt and detergent washes which have been found to alter the protein profile of the exosporium in both *B. cereus* (Todd *et al.*, 2003) and *B. anthracis* (Redmond *et al.*, 2004). Waller *et al.* (2005) identified a second collagen like glycoprotein of the exosporium, termed BclB. This protein is homologous to the *B. thuringiensis* glycoprotein ExsH and a 205 kDa glycoprotein within *B. cereus*. This 205 kDa glycoprotein was also found to contain an analogue of the BclA repeat GITGVTGAT; however, this motif is not found in *B. cereus* or *B. thuringiensis* proteins. Although the number of repeats and its relation to the structure of BclB has not been determined as with BclA, it was noted that an additional six repeats were present in the Ames strain compared to the Sterne strain from examination of the genome sequence (Waller *et al.*, 2005). More recently, from related work on the exosporium of *B. cereus*, ExsA was identified as an exosporium component (Todd *et al.*, 2003) and shown to be required for the assembly of the coat and exosporium on the surface of the spore (Bailey-Smith *et al.*, 2005). To date this protein has not been identified experimentally from *B. anthracis* spores; however, given the abundance of homologous exosporium proteins that have been identified, it is likely that, ExsA may have a similar role if present.

Knowledge of the synthesis and assembly of the exosporium is limited. From early studies on *B. cereus* the exosporium was only observed during the late stages of sporulation concurrent with assembly of the spore coat (Ohye and Murrell 1973). The first observation of deposition of exosporium components was also detected at Stage IV of sporulation. Assembly of the exosporium then continues until the structure is complete at the commencement of stage VI of sporulation (Ohye and Murrell, 1973). Following immunocytochemical examination of the formation of the exosporium and spore coat it was found that exosporium antigens assembled in the cytoplasm of the sporulating cell. In contrast, spore coat antigens were observed in the forespore during the early stages of septation (Short and Walker, 1975; Short *et al.*, 1977). At maturity the exosporium was observed as a complete layer surrounding the spore (Short and Walker, 1975; Short *et al.*, 1977).

Recent work conducted by Kang *et al.* (2005) suggests that the exosporium may have a fundamental role in protecting the spore from the host's immune response. It was shown that *B. anthracis* spores devoid of an exosporium were more susceptible to macrophage mediated killing than spores that possessed an exosporium. This work also demonstrated

that the target of the macrophage was not the dormant spore (shown by the use of a non-germinating mutant) but the germinated spore; however, results obtained regarding the fate of the spore within the macrophage are not consistent. For example, Welkos *et al.* (2002) suggest that an observed increase in germination within the macrophage may be related to increased elimination of the bacteria. In contrast Guidi-Rontani (2001) demonstrated the survival of the spore within the macrophage but showed that outgrowth was not possible within these cells.

#### 2.5.2.2 *The spore coat*

The structure and complexity of the spore coat varies depending on the species, but basically consists of an inner and outer layer (Holt and Leadbetter, 1969; Aronson and Fitz-James, 1976). As well as the two coat layers, a third layer has also been observed in some *Bacillus* species that lies between the inner coat and the cortex. It has been suggested that this could either be a further coat layer or the outer forespore membrane (Fujita, *et al.*, 1989; Sakae *et al.*, 1995). The spore coat can occupy up to 50% of the spore volume and consists mainly of protein, but also contains complex carbohydrates and lipids (Matz *et al.*, 1970; Beaman *et al.*, 1971). Evidence suggests that removal of the spore coat either by chemical methods or genetic mutation is not sporicidal. Spores lacking a spore coat retain the property of heat resistance; however, they do become sensitive to hydrogen peroxide, lysozyme and organic solvents (Gould and Hitchins, 1963; Gould *et al.*, 1970; Aronson and Fitz-James, 1971; Moir, 1981; Zheng *et al.*, 1988; Popham *et al.*, 1995; Jenkinson *et al.*, 1981). The spore coat also appears to be important for germination, as this structure allows the passage of smaller nutrient molecules to germination receptors located beneath the spore coat. Furthermore, enzymes that have possible roles in germination have also been found within the spore coat (Behravan *et al.*, 2000; Hullo *et al.*, 2001; Bagyan and Setlow, 2002). Morphological examination of the *B. anthracis* spore coat has, however, revealed that the coat is thinner than that observed in *B. subtilis* and although individual layers are less distinct, an inner and outer layer can still be observed (Roth and Williams; 1963; Gerhardt and Ribí, 1964; Hachisuka, *et al.*, 1966; Moberly *et al.*, 1966; Driks, 2002). Recent studies by Chada *et al.* (2003) and Driks (2003) have suggested that the spore may also act as a dynamic layer to allow for changes in volume.

A number of *B. subtilis* coat proteins have been identified and for many of these their roles in resistance and germination are predominantly unclear (Driks, 2002b). Of the *B. subtilis* spore coat proteins identified a proportion have been implicated in the assembly of the

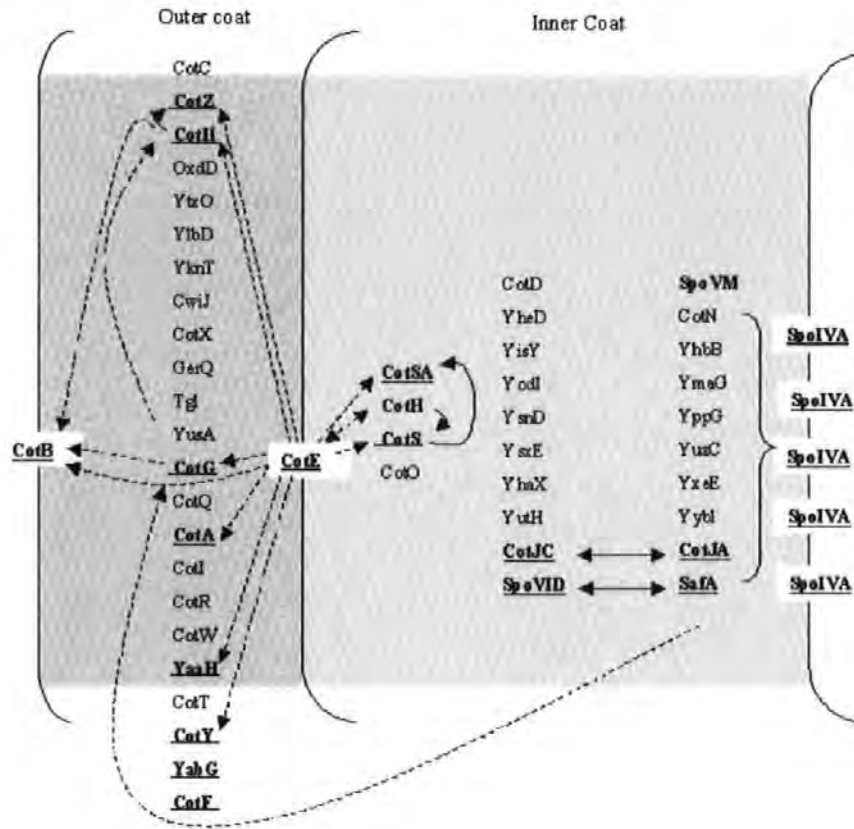
spore coat. However, for many coat proteins inference of function has been difficult, particularly as deletion mutants have often shown no observable difference in phenotype compared to the wildtype. More recently work conducted by Lai *et al.* (2003) identified 38 spore coat proteins of *B. subtilis* and a further 11 within *B. anthracis*. Comparative analysis of the *B. subtilis* and *B. anthracis* genomes shows that a number of homologues of known *B. subtilis* coat proteins are present within *B. anthracis* (Driks, 2002; Lai *et al.*, 2003). It has been hypothesised that, as many of these proteins are conserved between the two species, including the major morphogenetic proteins, a similar function and mechanism of coat assembly could be assigned to both species. However, it is notable that although proteins involved in correct assembly of the spore coat are present, many of the proteins required for the formation of the outer coat appear to be absent in *B. anthracis* (Driks, 2002; Lai *et al.*, 2003). Some of the predicted spore coat proteins (CotJC, CotB and CotY/ Z) have been shown to be present experimentally in spore coat extracts (Lai *et al.*, 2003). It has also been suggested that *B. anthracis* specific spore coat proteins are also present (Driks *et al.*, 2002a; Lai *et al.*, 2003). However, this may extend to the *B. cereus* group as with the recently identified major spore coat protein Cot- $\alpha$  (Kim *et al.*, 2004). It was found that this 13.4 kDa protein was expressed at stage IV of sporulation and appeared to be important for the resistance of the spore to toxic chemicals including hypochlorite, chloroform and phenol (Kim *et al.*, 2004). In the case of *B. subtilis* and *B. anthracis*, emerging evidence suggests that many of the functions of spore coat proteins require the interaction of a number of components (Bourne *et al.*, 1991; Thomas, 1993; Sacco *et al.*, 1995; Serrano *et al.*, 1999; Seyler *et al.*, 1997, Driks, 1999; Takamatsu *et al.*, 1998; Kuwana *et al.*, 2002; Bailey-Smith *et al.*, 2005; Lai *et al.*, 2003; Kim *et al.*, 2006). Therefore extensive characterisation of the spore coat is required to elucidate function of individual proteins.

#### Assembly of the spore coat

The primary stages of the assembly of the spore coat commence after septation of the vegetative cell and involve the interaction of a number of proteins under the control of  $\sigma^E$ . (Zheng and Losick, 1990; Roel *et al.*, 1992; Beall *et al.*, 1993; Stevens *et al.*, 1993; Zheng *et al.*, 1998). Coat assembly is believed to be predominantly a function of the mother cell due to the transcription of a number of different genes whose products may or may not be integral parts of the spore coat structure (Bourne *et al.*, 1991; Driks *et al.*, 1994; Sacco *et al.*, 1995; Driks *et al.*, 1994; Ishikawa *et al.*, 1998; Takamatsu *et al.*, 1998; Serrano *et al.*, 1999; Bagyan and Setlow, 2002). The majority of the coat proteins are synthesised after

activation of  $\sigma^K$  which occurs after forespore engulfment (Zheng and Losick, 1990; Aronson *et al.*, 1988; Cutting *et al.*, 1991; Zhang *et al.*, 1994; Sacco *et al.*, 1995; Naclerio *et al.*, 1996; Henriques *et al.*, 1997; Takamatsu *et al.*, 1998). In stage V of sporulation the spore coat and cortex layers form, and it is at this time that the spore's germination and resistance properties are observed (Dion and Mandestam, 1980; Jenkinson *et al.*, 1980). In *B. subtilis*, evidence suggests that the majority, of the outer and a proportion of the inner coat proteins are directed by CotE (Driks and Setlow, 2000; Kim *et al.*, 2006). However, other proteins such as SafA (YrbA) and SpoVID also appear to have an important role in coat assembly (Beall *et al.*, 1993; Ozin *et al.*, 2000, 2001; Takamatsu *et al.*, 2000; Kim *et al.*, 2006). In contrast, little is known about the structure and assembly of the *B. anthracis* spore coat.

A selected number of spore proteins and strong homologues within *B. anthracis* are shown in Figure 2.4.



**Figure 2.4** A model for spore coat assembly on an arc of the spore surface showing the possible interactions of proteins for which homologues exist in *B. anthracis*. A number of coat and coat associated proteins are denoted and those for which homologues exist within *B. anthracis* are bold and underlined. Known interactions are denoted by arrows with solid lines. Further possible interactions exist between a number of the proteins, determined from experimental studies conducted by Kim *et al.* (2006), denoted by arrows with dashed lines (Adapted from Driks, 2000 and Kim *et al.*, 2006)

### 2.5.2.3 The cortex and spore membranes

Two spore membranes of opposing structural polarity are formed as a result of forespore engulfment during sporulation (Wilkinson *et al.*, 1975), as discussed in section 2.5.1. In the mature spore these form the inner and outer spore membranes. The outer spore membrane is located below the spore coat, but observation by electron microscopy reveals that this structure is not continuous (Aronson and Fitz-James, 1976). Although the outer membrane appears to have an important role in spore formation, its role in dormancy is unclear. The cortex is a dense inner layer, consisting mainly of peptidoglycan that is similar, although not identical, to that found within the vegetative cell. Analysis of the

cortex peptidoglycan has revealed a unique structure not observed in the vegetative cell. Of note is the decreased amount of cross-linking of muramic acid residues (2.9%) in the dormant spore in contrast to 33 to 40% in the vegetative form (Atrih *et al.*, 1996; Popham *et al.*, 1996; Atrih and Foster, 1999). Furthermore, it has been observed that approximately half of all muramic acid residues are substituted with muramic  $\delta$ -lactam, required for hydrolysis of the spore peptidoglycan necessary for spore germination and outgrowth (Warth and Strominger, 1969; Sekiguchi *et al.*, 1995; Atrih *et al.*, 1996; Popham *et al.*, 1996; Meador-Patron and Popham, 2002). Cross-linking has been shown to be important for maintaining the dehydrated core allowing for heat resistance (Popham *et al.*, 1995; Popham, 2002). However, it has also been revealed that if the spore peptidoglycan is too highly cross-linked, degradation is difficult or not attainable; thus, spores are either incapable of germinating or the rate of outgrowth is reduced (Meador-Patron and Popham, 2002). Therefore, evidence suggests that muramic- $\delta$ -lactam has an important function in spore germination and outgrowth and the rate at which this occurs (Meador-Patron and Popham, 2002). Differences were also observed in the muramyl and N-acetylmuramic acid peptide side chains (Atrih *et al.*, 1996; Popham *et al.*, 1996).

There is a second layer of peptidoglycan below the cortex that is identical to that found in vegetative cells. This layer, also referred to as the germ cell wall, functions as the initial cell wall following germination (Moir *et al.*, 2002; Popham, 2002). Below the peptidoglycan layer is a second spore membrane, often termed the inner membrane. Following germination, this structure becomes the vegetative cell membrane, although its lipid composition and the protein to lipid ratio has been found to differ when the spore is in the dormant state (Freer and Levinson, 1967; Ellar, 1978). Proteins that have been identified in the *B. subtilis* inner membrane include; (a) GerAA and GerAC, both of which are required for germination (Hudson *et al.*, 2001); (b) SleB, a cortex lytic transglycosylase, involved in muramic- $\delta$ -lactam dependent cortex hydrolysis during germination (Chirakkal *et al.*, 2002) and (c) YpeB, required for the assembly and/or stability of SleB (Chirakkal *et al.*, 2002). Evidence suggests that the inner membrane is relatively impermeable to small hydrophobic and hydrophilic molecules and that the membrane lipids are immobile (Bertsch *et al.*, 1969; Cowan *et al.*, 2004; Cortezzo and Setlow, 2005).

#### 2.5.2.4 *The core*

Located at the centre of the spore is the core, which contains the spore DNA, ribosomes, the majority of spore enzymes required for germination, inactive metabolic enzymes necessary for outgrowth, and other small molecules. All of these components are immobilised in a lattice of salts of divalent cations (principally calcium, although magnesium, manganese and low concentrations of other divalent cations are also present), chelated in most likely a 1:1 ratio with pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA) (Murrell and Warth, 1965; Murrell, 1967; Gould, 1969). DPA accounts for up to approximately 20% of the dry core weight and exists predominantly in the spore core. DPA is thought to have a role in core dehydration, resistance of spores to wet heat and UV radiation, and maintenance of the spore in the dormant state, although the exact mechanism by which DPA confers these properties of the spore is unclear (Paidhungat *et al.*, 2000; Slieman and Nicholson, 2001; Nicholson *et al.*, 2002). Spore DPA and the associated divalent cations are secreted during the initial phase of germination and this has been used as a marker for spore detection (discussed further in section 2.6.4). Small acid soluble proteins (SASPs) are approximately 60-75 amino acids long and evidence suggests that they protect the spore DNA from a wide range of damage caused by UV radiation, chemicals, enzymatic attack and free radicals (Setlow, 1993; Setlow and Setlow, 1993, 1995; Fairhead *et al.*, 1994; Nicholson, 2000). SASPs are thought to bind to spore DNA in a manner that decreases the chemical reactivity of the DNA and causes a conformational shift from the normal right handed double helix to an torroidal structure (Setlow, 1988; 1992a; 1992b; Pogliano *et al.*, 1995). The spore has a significantly lower water content (~0.3-1.5g H<sub>2</sub>O per g dry weight) than that observed in the vegetative state (~4.0g H<sub>2</sub>O per g dry weight). The dehydrated state of the core is hypothesised to be required for the spore's heat resistant properties (Beaman and Gerhardt, 1986; Gerhardt and Marquis, 1989; Setlow, 1995;) and its ability to remain in a cryptobiotic state (Gerhardt and Marquis, 1989), believed to be due to decreased total: nicotinamide adenine dinucleotide (NAD); reduced nicotinamide adenine dinucleotide (NADH); nicotinamide adenine dinucleotide phosphate (NADP); reduced nicotinamide adenine dinucleotide phosphate (NADPH); and ATP concentrations in the spore compared to the vegetative cell. Furthermore, lower ratios of NADH:NAD and NADPH/NAD compared to the vegetative state have also been observed (Setlow and Setlow, 1977; Setlow, 1994).



## 2.6 Isolation and identification of *B. anthracis*

Vegetative cells of *B. anthracis* can be detected in a wide range of clinical specimens by specific staining of the capsule using a polychrome methylene blue stain (M'Fadyean stain), which was first demonstrated in 1903 (M'Fadyean, 1903). Typical colony morphology on blood or nutrient agar will be observed as being matt white/grey, flat and having a 'crushed glass' appearance and will be tacky. The colonies are often smaller than those observed with *B. cereus* or *B. thuringiensis* (Parry *et al.*, 1983). These methods are ideal with clinical specimens collected from infected individuals for a few hours after death. However, in other samples such as environmental samples, the numbers of *B. anthracis* present may be small and there will also be a natural background of other microorganisms. Of particular concern are those that are also within the *B. cereus* group. In this instance selective microbiological techniques are required to distinguish *B. anthracis* from other *Bacillus* species. *B. anthracis* can be distinguished from *B. cereus* and *B. thuringiensis* as it is: (a) non-motile; (b) catalase positive; (c) non-haemolytic or weakly haemolytic; and (d) sensitive to penicillin and the gamma phage. Furthermore, the organism does not hydrolyse urea, requires thiamine for growth, produces a glutamyl-polypeptide capsule and does not grow on chloralhydrate agar (Todar, 2004). Identification of *B. anthracis* using microbiological techniques is effective but time consuming. Therefore, a significant proportion of *B. anthracis* research is being directed at the development of specific, sensitive and reliable detection and diagnostic assays.

### 2.6.1 Genetic based methods of detection

Discrimination of members of the *B. cereus* group has proven particularly difficult at the genetic level due to the high levels of identity between genomes (Andersen *et al.*, 1996; Helgason *et al.*, 1990, 2000a, 2000b, 2004; Carlson *et al.*, 1994; Økstad *et al.*, 1999; Keim *et al.*, 2001; Ticknor *et al.*, 2001; Radnedge *et al.*, 2003; Priest *et al.*, 2004). Comparisons of 16S rRNA sequences and 16S-23S rRNA spacer regions have failed to distinguish between the three species (Ash *et al.*, 1991; Daffonchio *et al.*, 2000). Techniques such as amplified fragment length polymorphism (AFLP), variable number tandem repeats (VNTR), pulsed field gel electrophoresis (PFGE), multi-locus sequence typing (MLST) and multi-locus enzyme electrophoresis (MLEE) have also been used to identify different members of this group. More recent investigations have adopted the use of single nucleotide polymorphisms to identify individual isolates (Zwick *et al.*, 2005). This has

also been possible using multi-locus VNTR analysis (MLVA) (Keim *et al.*, 2005). The advantage of this type of approach is that identification of individual strains is possible, which may allow for identification of the original source.

A range of assays have been developed for the detection of *B. anthracis* using the polymerase chain reaction (PCR) (Beyer *et al.* 1996; Patra *et al.* 1996; Sjöstedt *et al.* 1996; 1997; Bode *et al.*, 2004). However, there are problems with this approach. Most rapid real time PCR assays have relied on the use of the virulence determinants encoded within pXO1 and pXO2 (Ramise *et al.*, 1996; Bell *et al.*, 2002; Ellerbrook *et al.*, 2002). Given the identification of a *B. cereus* strain also carrying pXO1 (Hoffmaster *et al.*, 2004), the presence of some isolates cured of these plasmids (Turnbull *et al.*, 1992; Ramise *et al.*, 1996) and the possibility of transfer, loss or purposeful genetic modification (Baillie, 2005), complete reliance on the presence of these plasmid encoded genes is not ideal. Therefore, identification of other genes to be used in a multiplex format would increase the confidence of the results of an assay. However, discrimination of *B. anthracis* from other members of the *B. cereus* group via chromosomally encoded genes is exceptionally difficult, although some genetic differences have been reported (Cherif *et al.*, 2003). A number of groups have recently reported the use of real time PCR in the specific detection of *B. anthracis* through the identification of single point mutations in the *rpoB* and *gyrA* genes (Qi *et al.*, 2001; Drago *et al.*, 2002; Hurtle *et al.*, 2004). However, the specificity of such assays is regarded by some groups as being very sensitive to slight alterations in assay conditions, resulting in false positive results. Work conducted by Elbrook *et al.* (2005) identified a sequence unique to *B. anthracis* by subtractive hybridisation to develop a real time PCR assay with 100% specificity against a range of *Bacillus* strains. However, further testing to ensure detection of a larger number of *B. anthracis* strains is required. In general, PCR assays are ideal for clinical samples where detection of DNA from the vegetative form is possible; however the detection of spores, which would be required for environmental monitoring, requires germination and/or outgrowth or disruption of the spore itself to release DNA, something that has proven difficult (Ryu *et al.*, 2003). Although a low amount of the total DNA is suggested to be associated with the spore surface, reliance on this renders the assay unquantitative and relatively insensitive. This factor has a second disadvantage in that highly purified spores may not have any DNA associated with the outer spore surface, and hence assays that have not been rigorously tested may be ineffective, resulting in false negative results. Environmental samples may require concentration after collection, due to the small sample volumes required for a PCR

assay, and possibly some form of sample processing, not only to extract the DNA but to eliminate any potential inhibitors of the PCR reaction (Alvarez *et al.*, 1995; Kuske *et al.*, 1998). There is an opinion that these assays require further development to be reliable, accurate and sensitive enough for purpose (Webb, 2003). Furthermore, PCR often requires the use of trained personnel unless incorporated into a fully automated system, and the reagent requirement is relatively high, this increases costs and logistical problems, particularly for continuous environmental sampling. The report of the development of a universal biosensor for the detection of pathogenic microorganisms, including *B. anthracis* was reported by Baeumer *et al.* (2004) using an oligonucleotide hybridisation assay. However, although showing promise, this assay may be limited to laboratory confirmation due to the requirements for incubations at elevated temperature and precise buffer requirements for hybridisation. Furthermore, this group also acknowledges that the format of the assay requires simplifying. However, advances in microarray technology and data processing may enable this type of assay to be fielded in the future.

The sequencing and annotation of the *B. anthracis* Ames genome and comparison with related bacteria such as *B. cereus* (Ariel *et al.*, 2002), has allowed for the identification of further putative virulence genes. Microarray analysis conducted by Sergeev *et al.* (2005), identified the presence of putative virulence factor genes within *B. anthracis* and other members of the *B. cereus* group which have the potential for use in genetic based detection and diagnostic assays. However, the presence of these genes does not indicate that they are transcribed, highlighted by the fact that many of these genes were also found to be present in the *B. subtilis* negative control (Sergeev *et al.*, 2005).

This highlights the fact that gene transcription, and thus detection of the gene products may be invaluable in discriminating *B. anthracis* from other members of the *B. cereus* group. Genetic methods of identification would appear to be invaluable as confirmatory assays, and of particular use in specific strain identification (Levy *et al.*, 2005) that could be used for forensic analysis within a relatively short time period compared to standard microbiological techniques. However, real-time detection of an environmental hazard by continuous monitoring is not practical at present (Webb, 2003).

## 2.6.2 Recognition element-analyte recognition based methods of detection

A recognition element is any molecule capable of binding a target analyte, there are many different types of recognition elements, discussed in further detail in 2.8. The most common forms of recognition element used for detection of an analyte from a given species are antibodies. Immunoassay-based techniques, being quicker than standard microbiological techniques, show promise for rapid real time detection. Whilst more established assay formats such as ELISA are time consuming, other applications such as lateral flow assays or incorporation of the antibodies into other biosensors are more rapid (discussed in further detail in section 2.7). However, assays that rely on the use of recognition elements have encountered problems in sensitivity and in the generation of an antibody or other recognition element that is specific to *B. anthracis*. In the case of antibodies, polyclonal (pAbs) and monoclonal (mAbs) antibodies have been produced with a view to reducing the cross-reactivity observed with other *Bacillus* species. In the case of polyclonal antibody, selective adsorption has been utilised with varying degrees of success (Lamanna and Eisler, 1960; Fluck *et al.*, 1977; Phillips *et al.*, 1983). In one case the complete removal of cross reactivity to closely related species was observed (Phillips and Martin, 1988). However, given the variation in antibody specificity that would exist from antibodies raised both between animal species and within different animals of the same species, this would not be suitable for reproducible specificity and sensitivity of reagents. To overcome this, murine mAbs offer a more suitable alternative (as discussed section 2.10.2.2). There are limited reports of the production of species specific mAbs to *B. anthracis* spores (Phillips *et al.*, 1988; Turnbough and Kearney, 2002), that demonstrated low levels of cross reactivity with *B. cereus* and *B. thuringiensis* have been reported and the epitopes or antigens that they recognise have not been detailed. Early research into the production of antibodies against *Bacillus* spores focused on the inoculation of animals with live spores. To eliminate any immune response to vegetative cell antigens, penicillin was administered (Lamanna and Eisler, 1960; Norris, 1962). However, the use of live spores did incur problems including an immune response to vegetative cell antigens if prophylaxis was unsuccessful, and death of the immunised animal. This led to the immunisation of animals with spores inactivated with heat, formaldehyde or irradiation to produce polyclonal (Fluck *et al.*, 1977; Phillips and Martin, 1983; 1988; Phillips *et al.*, 1983; Phillips *et al.*, 1988; Stopa, 2000) and monoclonal (Phillips *et al.*, 1988; Dang *et al.*, 2001; Turnbough and Kearney, 2002; Steichen *et al.*, 2003) antibodies. However, this approach is not ideal as methods of inactivation can cause differences in the ability of resulting antibodies to recognise live spores. Thus, it has been suggested that inactivation

methods may alter antigens on the spore surface compared to the live spore, for example through denaturation (Phillips *et al.*, 1988; Dang *et al.*, 2001). With the use of both inactivated and live spores, resulting antibodies still demonstrate cross reactivity with other *Bacillus* species due to the presence of homologous proteins (Lamanna and Eisler, 1960; Norris, 1962; Phillips *et al.*, 1983; Fluck *et al.*, 1977; Longchamp and Leighton, 1999; Stopa, 2000).

More recent research suggests that the majority of monoclonal antibodies raised against whole *B. anthracis* spores recognise the glycoprotein BclA, a protein which also has a homologue within *B. cereus* (Steichen *et al.*, 2003; Redmond *et al.*, 2004). The identification of a unique marker or epitope of a known protein is essential in obtaining reagents with the required sensitivity and specificity as well as increasing confidence in positive identification.

In addition to traditional antibodies, other forms of recognition element have been produced and utilised for the detection of *B. anthracis* spores. The advantages of the use of such receptors are discussed in sections 2.8-2.11. One such type of recognition element that has been employed are peptide recognition elements, usually produced by phage display. These recognition elements have had a limited amount of success with regard to the detection of *B. anthracis* spores. Brigati *et al.* (2004) reported the production of peptide recognition elements from a landscape phage display library. Although these recognition elements demonstrated increased specificity to *B. anthracis* spores, cross reactivity with *B. cereus* and *B. thuringiensis* was still observed. Williams *et al.* (2003) describe the specific identification of *B. anthracis* through the use of pattern recognition with two peptides; one which binds to *B. anthracis* and a small subset of *B. cereus* and *B. thuringiensis* strains and the other which did not bind to *B. anthracis*. In this case, the exact epitope to which the peptides bound has not been determined.

Lectins have also been used for the discrimination of *B. anthracis* from other *Bacillus* species (Cole *et al.*, 1984). This research was predominantly focused on vegetative cells due to the presence of a galactose containing polysaccharide on the outer surface of *B. anthracis*. However, the observation that spores have lectin binding sites was hypothesised

to be due to: (a) retention in an unmodified form, (b) penetration of the receptors through the external layers, (c) the synthesis of spore-specific lectin receptors of similar composition to those observed in the vegetative state, or (d) vegetative cell contamination. From this work, differentiation of spores was possible as *B. anthracis* spores showed strong binding to APA, GSA-1, RCA-1, RCA-II and SBA<sup>1</sup>. No binding to these lectins was observed in *B. subtilis*. Only one strain of *B. cereus* (6464) demonstrated binding to APA and only weak binding was observed with APA, RCA-1 and RCA-II (*B. mycoides* 6462) and APA, GSA-I, RCA-I, RCA-II with strong binding to SBA<sup>1</sup>. This demonstrates that although the lectins tested are not completely specific, discrimination of *B. anthracis* is possible through pattern recognition. More recent work conducted by Tarasenko *et al.* (2004) used the interaction of carbohydrates for the detection of *B. anthracis* spores by employing glycoconjugates as the recognition elements. In biological systems, glycoconjugates have a range of important roles such as signalling, adhesion and/or recognition (Bovin, 1997; Feizi, 2000). More significantly, receptors for microbial pathogens on host cells and the structures they recognise are often glycoconjugates (Feizi, 2000), leading to a hypothesis that interactions are via carbohydrate/carbohydrate binding (Misevic and Burger, 1993; Stewart and Boggs, 1993; Misevic and Popescu, 1995; Zhu *et al.*, 1995; Koshy and Boggs, 1996;). Furthermore, the binding of carbohydrates to glycoconjugates has been established for a number of cell surface proteins, such as those involved in adhesion (Bovin, 1997; Rye and Bovin, 1997; Houseman and Mrksich, 2002). Various studies have been aimed at mimicking spore recognition (Tarensenko *et al.*, 2002; Ji *et al.*, 2003; Williams *et al.*, 2003; Knurr *et al.*, 2003; Kazakov *et al.*, 2004). In the work conducted by Tarensenko *et al.* (2004), the glycoconjugate binding capacities of *B. cereus*, *B. thuringiensis*, *B. pumilus* and *B. subtilis* were observed. Again, differences could be discerned through pattern recognition of the spore to different carbohydrates (disaccharides). It was hypothesised that binding of the disaccharides was through recognition of a monosaccharide on the outer surface; however, this has not been proven. Of note was the fact that *B. cereus* and *B. thuringiensis* showed stronger binding than *B. subtilis* and *B. pumilus*, possibly due to the larger exosporium in the *B. cereus* group. This indicates that the exosporium may have an important involvement in carbohydrate binding and thus potentially host cell recognition, supporting the observations of the role of the exosporium in macrophage uptake shown by Kang *et al.* (2005).

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<sup>1</sup> APA- *Abrus precatorius* (specificity for  $\beta$ -D-Gal $\gt$  $\alpha$ -D-Gal), GSA-I – *Griffonia simplicifolia* (specificity for  $\alpha$ -D-Gal  $\gt$   $\alpha$ -D-GalNAc, RCA-I – *Ricinus communis* (specificity -  $\beta$ -D-Gal $\gt$  $\alpha$ -D-Gal), RCA-II – *Ricinus communis* (specificity for  $\beta$ -D-Gal $\gt$  $\alpha$ -D-Gal), SBA – *Glycine max* (specificity -  $\alpha$ -D-GalNAc $\geq$  $\beta$ -D-GalNAc $\gg$  $\alpha$ -D-Gal)

Other recognition elements that have been used for the detection of *B. anthracis* spores include single chain antibodies, carbohydrates and aptamers (Bruno and Kiel, 1999; Zhou *et al.*, 2002; Tarasenko *et al.*, 2004). These are discussed in more detail in sections 2.8 and 2.11. However, none of these novel recognition elements represent a truly specific recognition element to *B. anthracis*, as varying degrees of cross reactivity are observed with closely related species. Thus, a truly specific recognition element of this type has not been described to date.

### **2.6.3 Spore-specific markers for recognition element based detection**

An ideal detection target for a recognition element-based assay would be unique, abundant, surface located, expressed under all growth conditions and be essential, such as house keeping genes, or required for virulence. As recognition element based detection assays are predominantly antibody based, there is an inherent reliance on the identification of immunogenic targets. Evidence from early research predicted that antigens located within the spore would be useful in the identification of and discrimination of *Bacillus* species (Norris, 1962). However, this has proven to be more complex than originally anticipated due to the close genetic relationship between *B. anthracis*, *B. cereus* and *B. thuringiensis*. As all of these are environmental organisms, and with *B. thuringiensis* being used widely as an insecticide, the degree of relatedness has implications for the production of specific recognition elements for environmental monitoring. To date only one *B. anthracis* specific antigen has been identified, described by Park *et al.* (2000). However, the identity of the antigen was not reported. The advantages of other recognition elements, such as peptides or synthetic recognition elements, are that it is possible to generate recognition elements against non-immunogenic targets (discussed in more detail in section 2.11-2.12). Thus wider range of detection targets can be considered.

The exosporium is the primary barrier between the spore and the external environment, therefore targets located within the exosporium could be useful in the understanding of *B. anthracis* pathogenesis and the development of targets for detection and diagnostic purposes. However, only recently have a proportion of exosporium components been identified and of these none have been found to be specific to *B. anthracis*, although some

are specific to the *B. cereus* group (Steichen *et al.*, 2003; Todd *et al.*, 2003; Redmond *et al.*, 2004; Lai *et al.*, 2004; Liu *et al.*, 2004). To date only a few of these components have been characterised (as discussed in section 2.5.2.1). Further consideration needs to be given to the nature of a spore specific marker; for example, the identification of unique regions or signatures within a protein and expansion of prospective markers for specific identification beyond those located only on the outer surface may be possible. Once a specific protein or region has been identified these could be applied to a range of detection platforms that have in the past been unsuccessful in specific identification of *B. anthracis* spores, such as immunochromatography (lateral flow assays), flow cytometry (Stopa *et al.*, 2000; Enserink *et al.*, 2001) or other biological sensors which are discussed in section 2.7.

Spore-specific carbohydrates could also be utilised for the detection of spores and possibly for discrimination at the species level. The first spore specific carbohydrate to be reported was rhamnose from the exosporium of *B. cereus* (Matz *et al.*, 1970). The presence of galactosamine and galactose on the spore surface was demonstrated through the use of lectins by Cole *et al.* (1984). Later work has reported 3-*O*-methyl rhamnose and galactosamine isolated for *B. anthracis*, *B. cereus* and *B. thuringiensis*. The latter two species can be discriminated from *B. anthracis* by the presence of fucose and 2-*O*-methyl rhamnose by the use of GC-MS (Fox *et al.*, 1993; Wunschel *et al.*, 1994). Both the spores and vegetative cells of these species were found to contain ribose, glucose, galactose, glucosamine, mannosamine and muramic acid. Muramic acid and glucosamine are present within peptidoglycan and galactose and mannosamine were hypothesised to be present in peptidoglycan-polysaccharide in vegetative cells. The presence of these components within spores is believed to be due to vegetative cell debris or residual cells within spore preparations (Fox *et al.*, 2003). The presence of a spore specific carbohydrate (3-*O*-methyl-rhamnose) was also noted within *B. subtilis*; however, this differed from *B. anthracis* due to the presence of a rhamnose isomer, quinovose (Wunschel *et al.*, 1994). Examination of the ultrastructure of *B. anthracis* spores using a carbohydrate stain showed staining of the hairy-nap and basal layers, suggesting these all have a strong carbohydrate component. Although much of this work was based on GC-MS profiling, the complexity of the analysis would be too problematic for a fieldable system; however, further work characterising the glycoproteins of *Bacillus* species could be valuable for many biodetection platforms.



#### 2.6.4 Other methods of detection

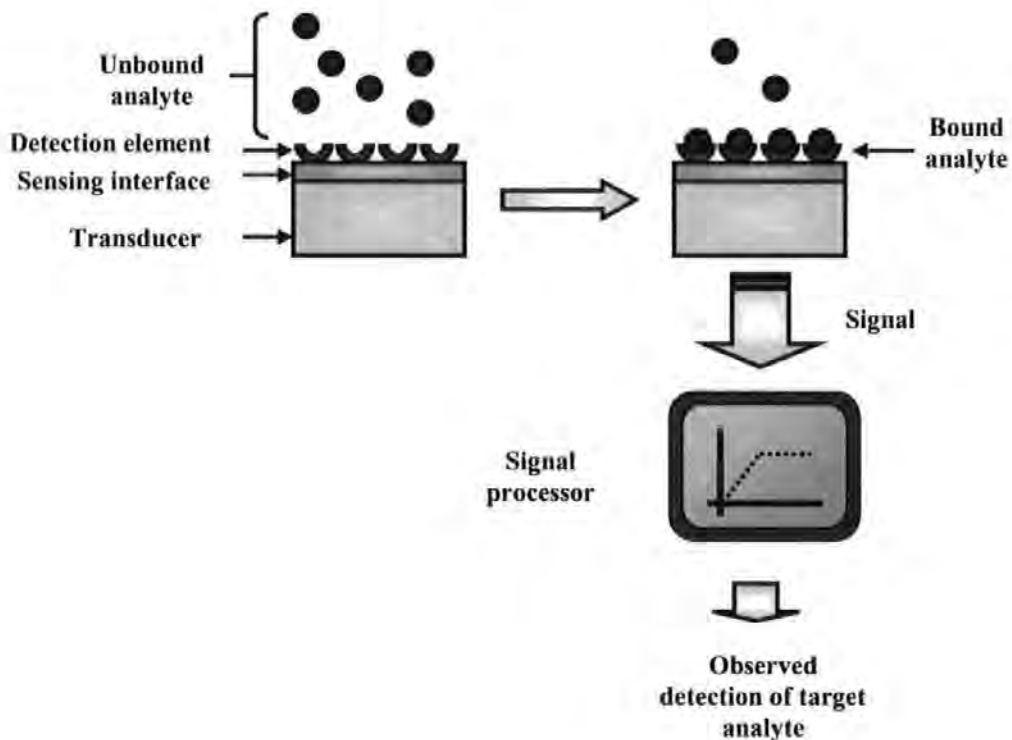
Spore specific markers can also be used for non-recognition element based identification for example the identification of a particular spore component such as DPA through a chemical reaction or spectrometry-based methods may be used. DPA has also been utilised as a marker for generic spore detection. As discussed in section 2.5.2.4, in the minutes following spore germination DPA is secreted, which can be triggered artificially under laboratory conditions. DPA can be detected using a number of methods; one of the most promising methods is based on the fluorescence of DPA-lanthanide metal complexes. Terbium has been found to give the most promising resulting in detection limits of  $\sim 10^4$  cfu/ml for bacterial spores (Hindle and Hall, 1999). Although this method shows promise it is only able to discriminate spores from vegetative cells and thus may not be ideal for environmental monitoring specifically for *B. anthracis*. In addition, the requirement for 70° C heat shock and subsequent germination with L-alanine cause a delay in the time required for positive identification.

Mass spectrometry based methods have shown promise due to their ability to rapidly process and identify proteins of a given organism under laboratory conditions. Dickinson *et al.* (2004) were able to discriminate the spores of different bacterial species using matrix-assisted laser desorption ionisation-time-of-flight mass spectrometry (MALDI-TOF MS). In the past, one of the predominant challenges in the use of this technique was the extraction of the spore proteins prior to mass spectrometry analysis, and have included infrared laser irradiation (Ryzhov *et al.*, 2000, Ullorn *et al.*, 2001), corona plasma discharge (Hathout *et al.*, 1999), sonication, or the use of chemical methods such as 5% Trifluoroacetic acid (TFA) (Hathout *et al.*, 2003) or 1M HCl. The problems with these approaches are the time required for sample processing and the fact that peaks greater than 10 kDa are often not observed. Therefore, many of the reports of these methods focus on the use of SASPs as biomarkers, such as those described by Hathout *et al.* (2003). In this instance, differences were observed in the masses or tryptic peptide maps obtained from major and minor  $\alpha/\beta$  and  $\gamma$ -type SASPs from *B. anthracis* and *B. cereus/B. thuringiensis* but not between the latter two species. This method would be unsuitable for rapid detection as the complexity and length (90 min) of the extraction method would make it unsuitable for fieldable use. The method described by Dickinson *et al.* (2004) used a simpler and more rapid solvent extraction, and described sample preparation of a couple of minutes, but was unable, as yet to distinguish between members of the *B. cereus* group. Furthermore, this method would require extensive automation for real time environmental

monitoring with significant sample clean up and is probably of more use as a confirmatory technique.

## 2.7 Biosensors

In a broad context a biosensor can be considered as any sensor that utilises a biological moiety for detection (Piervincenzi *et al.*, 1998), but has often been extended to include the detection of biological species (Lowe, 1999; Marose *et al.*, 1999). At the most basic level, a biosensor typically consists of a detection element (for example an antibody or enzyme), a signal transduction event, and a signal processor (Byfield and Abuknesha, 1994). The detection event may be direct; for example, the detection of analyte binding to a recognition element; or indirect, where detection of a second labelled moiety or the product of a secondary reaction is required. The interaction between the detection element and analyte is converted into an observed detection event by signal transduction. An example of a simple biosensor using direct detection is shown in Figure 2.5.



**Figure 2.5** Example of the principal components of a biosensor using direct detection.

The signal measured is due to a change of a particular parameter, such as capacitance, resistance, mass or fluorescence. Examples, but not an exhaustive list, of different types of biosensor that have been used for bacterial detection, divided on the basis of the method of transduction, are shown in Appendix 2.1. A number of comprehensive reviews of biosensor technologies have been conducted (Luppa *et al.*, 2001; Iqbal *et al.*, 2000; Ivnitski *et al.*, 1999). Biosensors can be utilised for the detection and identification of a range of analytes, from low molecular weight species, for example ions, to larger particulates, such as viruses and bacteria. Typically, detection utilising biosensors is specific for a target analyte, but generic biosensors have also been developed. Dependent on the final application, desirable properties for a biosensor may include that the system is essentially reagentless, robust, rapid and capable of detection in defined conditions with a sensitivity and specificity suitable for the purpose of the assay. One of the largest groups of biosensors utilised for the specific detection of target analytes through the incorporation of recognition elements are optical biosensors, which often provide an essentially reagentless system capable of near real-time detection of a target analyte (Setford, 2000). Commonly, these sensors are based on evanescent wave technology such as SPR, the resonant mirror or fibre optic waveguides, all of which have been used successfully for the detection of a wide range of analytes (Medina *et al.*, 1997; Watts *et al.*, 1994 Anderson *et al.*, 2000). More recently, novel methods have been explored such as the use of whispering gallery modes in microspheres (Arnold *et al.*, 2003). The other most common transduction methods which incorporate recognition elements are piezoelectric such as surface acoustic wave devices, quartz crystal microbalances, mechanical cantilevers (Wu *et al.*, 1990; Liss *et al.*, 2002; Ilic *et al.*, 2004) or electrochemical, including potentiometric and amperometric methods (Wang *et al.*, 1997; Uithoven *et al.*, 2000). Although many of these biosensor technologies utilise recognition elements, other methodologies exist for specific detection without the use of a recognition element. Examples include spectroscopic methods, such as Raman spectroscopy (Nelson *et al.*, 1992). However, one significant limitation of spectroscopy based techniques has been the analysis of spectra often obtained from complex particulates such as bacteria, to allow for identification of the desired target (Rossi and Warner, 1985; Jarvis and Goodacre, 2004). A further desirable function of a biosensor which uses a recognition element for specific detection is the linkage of the binding event to signal transduction (Hellinga and Marvin, 1998). In the design of a biosensor, important considerations include the transduction mechanism, the nature of the analyte, the specific recognition element (if one is to be used) and the interaction between the two, which may include, for example, steric effects and diffusion. Many of these parameters are determined by the nature and use of the detection element

(the specific recognition element) utilised within the sensor. In order to obtain reliable, sensitive and rapid detection of a pathogen any one of these parameters could be evaluated.

## 2.8 Recognition elements

The most common natural molecules used in the specific detection of pathogenic microorganisms are antibodies, although other elements include enzymes, peptides, lectins, carbohydrate receptors, complementary nucleic acid probes and aptamers (Kiefel and Itzstein, 2002; Hone *et al.*, 2003; Williams *et al.*, 2003; Baeumner *et al.*, 2004; Kirby *et al.*, 2004). More recently, elements such as whole cells and tissues of plants and animals have been utilised, such as genetically engineered pathogen specific B-cell lines which have been used to produce the CANARY sensor (Rider *et al.*, 2003). The use of genetically engineered bacteria has also been described for detection and used in an array format (Kuang *et al.*, 2004). Non-natural recognition elements can also be used in the detection of biological species, such as synthetic recognition elements. Examples of these include chemical compounds, synthetic peptides or molecularly imprinted polymers (Kodadek *et al.*, 2004; Zimmerman and Lemcoff, 2004). Chemical compounds and synthetic peptides are usually isolated through the production and screening of large combinatorial libraries (Lam *et al.* 1991). Molecularly imprinted polymers are produced as functional and cross-linking monomers which are co-polymerised in the presence of the target of interest, creating a molecular imprint that may have the potential to have similar affinity and selectivity to antibodies (Vlatakis *et al.* 1993; Haupt and Mosbach, 1999). Although synthetic approaches are often attractive, as the recognition elements are inherently robust and relatively easy to produce, one major disadvantage to their use has been obtaining a recognition element with the same binding properties (for example affinity) of an antibody (Kodadek *et al.*, 2004). A further development within biosensors that has been investigated more recently is incorporation of the signal transduction into the recognition element (Bark and Hahn, 2000; Jiang *et al.*, 2004). Alternatively, direct signal transduction can be achieved through a detectable change (e.g., a visible colour change) in the surface after binding of the target analyte to an appropriate recognition element; this has been termed a 'smart' or 'self-reporting' surface (Englebienne, 1999; Song *et al.*, 2002).

## 2.9 The use of antibodies in biosensors

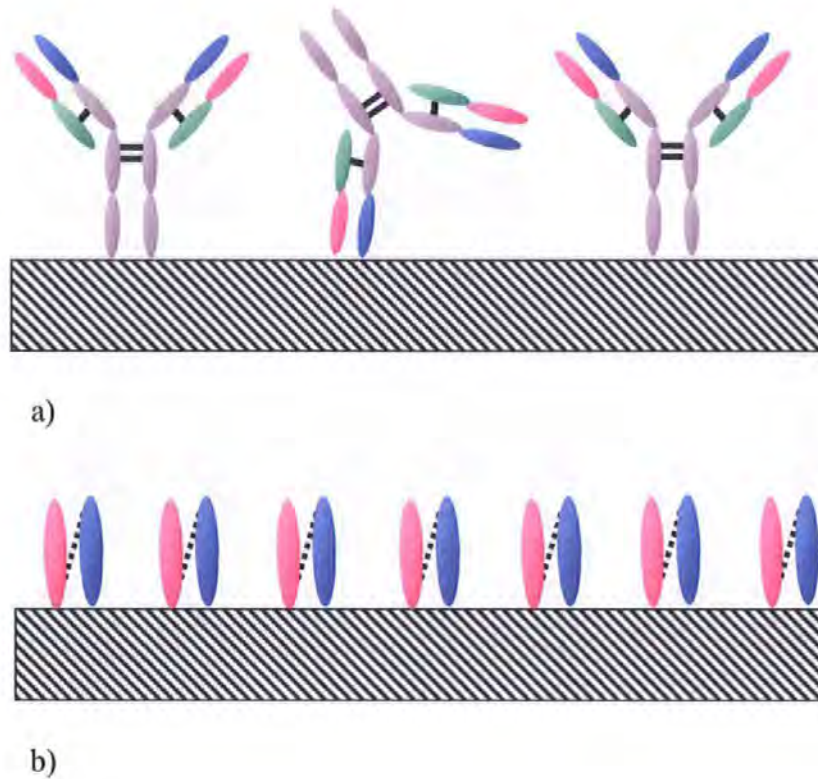
Antibodies represent a readily available natural source of molecules that have the capacity to recognise a wide range of target analytes ranging from defined and characterised markers such as specific proteins to complex undefined larger particulates such as bacteria (Hellings and Marvin, 1998). As a result, antibodies have been utilised extensively on a wide range of detection platforms, including biosensors (Appendix, Table A1.1). The specificity and sensitivity of these immunoassay based technologies are limited by the quality of the antibody produced and the selection of the target for recognition. Production of poly- and monoclonal antibodies by conventional methods has provided a platform that is the basis of many detection and diagnostic technologies. Although antibodies have been widely exploited for use in a range of biosensors, use of these natural molecules has often required the modification of detection technologies to optimise performance. For example, this may involve the use of additional reagents (Fratamico *et al.*, 1998), optimisation of assay conditions (Studentsov *et al.*, 2002) or the development or modification of new or existing technologies (Perkins and Squirrel, 2000). Furthermore, these natural recognition elements can be sensitive to particular assay conditions (Shone *et al.*, 1985) and may not provide the sensitivity and specificity required. When used in a biosensor, sensitivity is determined not only by the inherent limitations of the antibody to bind to a given target, but may also be affected by the number of available binding sites dictated by the density of recognition element immobilised and orientation onto a sensor surface (Hock *et al.*, 2002). Further consideration also has to be given to parameters such as steric hindrance, diffusion of the target analyte and the ability of the surface and recognition element to be effectively reused or regenerated (Leidberg and Göpel, 1997). When antibodies are used within biosensors it is frequently required that they are immobilised onto a solid support at the sensing interface. A number of strategies exist for antibody immobilisation onto a sensor surface as shown in Table 2.1.

Method	Examples	Orientation of Ligand	Leaching	Other considerations
Physical adsorption	Use of a suitable surface for protein retention e.g. nitrocellulose or prefabricated surfaces using techniques such as corona ionised gas or glow discharge	Random	Can be problematic	Regeneration of ligand difficult
Covalent attachment	Use of primary amines, thiols and carbohydrate groups on the molecule to be immobilised	Can be specific if the ligand can be modified or engineered		Regeneration of ligand easier than when the antibody is immobilised by other methods, dependent on stability of antibody to regeneration conditions
Biospecific (anti-ligand to ligand)	Protein A, Protein L, Protein G, anti-species antibody	Specific orientation through the Fc portion of the antibody	Can be problematic	Regeneration of ligand difficult
Biospecific (specific tag and interaction with another species)	Biotin/Avidin, His-Tag/Metal Chelate	Can be specific if the ligand can be modified/engineered	Can be problematic	Regeneration of ligand difficult
Entrapment	Within a polymer, gel or membrane	Non-specific but can be coupled with covalent attachment		Regeneration of ligand difficult and there may be constraints on binding and regeneration imposed by diffusion. Entrapment may increase stability

**Table 2.1** Antibody immobilisation strategies

The choice of strategy employed will be dependent on the nature of the sensing interface. Techniques such as surface adsorption are time consuming. Often the density of antibody loading is relatively low and can be unstable over extended time periods, or in harsh buffering conditions, such as those required for regeneration (elution of the bound analyte) (Ahluwalia *et al.*, 1992). Approaches most commonly used for antibody immobilisation onto a biosensor involve covalent attachment, for which a wide range of sensing interfaces have been employed, including self-assembled monolayers, lipid bilayers, silanised glass surfaces, gold, functionalised magnetic beads and dextran (Shriver-Lake, 1998; Egdage and Wilson, 1998; Fägerstam *et al.*, 1992; Nyquist *et al.*, 2000). A significant advantage of covalent methods of immobilisation is that the antibody is also stable in high buffer flow rates and after a number of regeneration cycles due to the stability of the bond formed between the antibody and a functional group (Catimel *et al.*, 1997; Howell *et al.*, 1998).

Covalent attachment also allows the immobilisation of high concentrations of recognition element enabling maximum loading of the sensing region with functional binding sites. High surface loading must, however, be balanced with steric hindrance of analyte binding that may occur if the surface density of the recognition element is too high (Vijayendran and Leckband, 2001; Brogan *et al.*, 2003). Furthermore, as with some of the other techniques, covalent attachment may not allow for specific orientation of the antibody to exposed binding sites; such as immobilisation *via* amine groups (Kortt *et al.*, 1997). Amino groups that can be used for non site-directed covalent attachment are located all over the antibody. Therefore, immobilisation occurs in a random orientation so the antibody may either be; (a) fully exposed and available for binding; (b) partially exposed (but there may be steric problems); or (c) not exposed and not available for binding (Figure 2.6) (Nisnevitch and Firer, 2001). The advantage of the use of engineered recognition elements such as Fab fragments or single chain antibodies (scFv) is that site directed attachment to a biosensor surface can be achieved allowing all binding sites to be available. Subsequently a significant increase in apparent affinity and assay sensitivity may be observed (Kortt *et al.*, 1997). Furthermore, the use of smaller molecules such as scFv will increase the density of antibody that can be immobilised onto a surface compared to that achievable with traditional monoclonal or polyclonal antibodies, also allowing for an increase in assay sensitivity (Figure 2.6).



**Figure 2.6** Immobilisation using a) a non-directed coupling method onto a sensor surface using traditional antibodies and b) a site-directed coupling method using scFv.

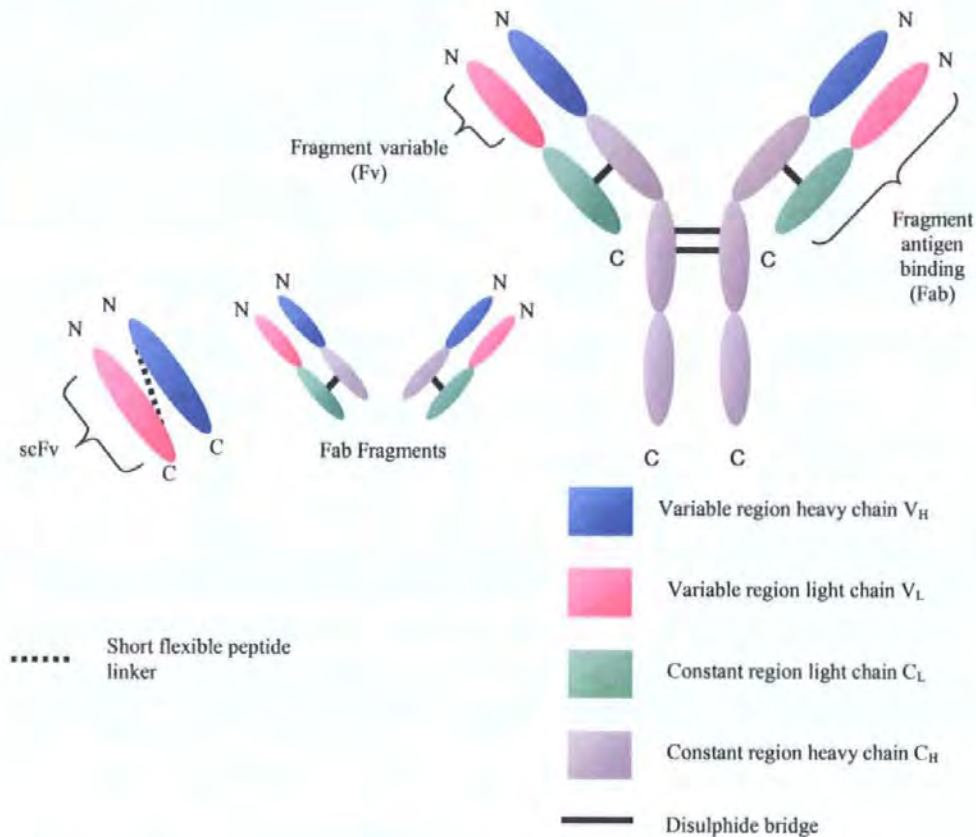
### 2.10 ‘Traditional’ antibodies

In order to obtain a full understanding of the limitations of traditional antibodies and the potential of engineered antibodies, an explanation of antibody structure and production *in vivo* is required.

Immunoglobulins (Igs) are tetrameric structures consisting of two heavy chains (m.w. ~55 kDa, 440-550 aa) and two light chains (m.w. ~25 kDa, 220 aa). The light and heavy chains consist of constant and variable regions and show patterns in the primary structure. The constant regions are located at the C-termini of the heavy and light chains ( $C_L$  or  $C_{H1}$ ,  $C_{H2}$  and  $C_{H3}$ ), the structure and sequence of these are conserved within an antibody sub-class. The light chain is linked to the heavy chain via a disulphide bridge towards the N-terminus of the heavy chain (Figure 2.7). The two heavy chains are not only linked via a disulphide bridge at the hinge region, but also through non-covalent interactions at the C termini



(Figure 2.7). Within the constant region of a defined isotype the amino acid sequence will be relatively well conserved. The variable regions of the heavy and light chains show little identity within any isotype. Within each variable domain are four conserved framework sequences composed of two antiparallel  $\beta$ -sheets (Poljak *et al.*, 1973). However, the greatest variation in amino acid sequence is observed at the antigen binding sites located at the N terminus - this is the variable region (Fv). Contained within the variable region of both the heavy and light chain are three non contiguous regions which dictate the specificity of a particular antibody. The specificity is governed by the amino acid sequence that forms six polypeptide loops constituting the complementarity determining regions (CDRs), three from  $V_H$  (CDR H1-3) and three from  $V_L$  (CDR L1-3) which together form the antigen binding site (Kabat and Wu, 1971; Jones *et al.*, 1986). The regions  $V_H$ ,  $V_L$  and  $C_L$  can be cleaved to produce the fragment antigen binding (Fab), discussed in more detail in section 2.11.1.  $V_H$  and  $V_L$  can be linked by a short peptide linker from the C terminus of one domain with the N terminus of the other, to form a single chain Fv or scFv (Breitling and Dübel, 2000).



**Figure 2.7 The basic structure of IgG showing the Fv region**

The immunoglobulin molecule has two identical light and heavy chains; however, the molecule adopts an asymmetric conformation. This fact demonstrates the ability of the two fragment antigen binding (Fab), portions to be present in different conformations in relation to each other and the Fc domain. The production of Fabs is discussed in further detail in section 2.11.1. It has been suggested that the flexibility of the IgG molecule is critical for its functionality.

### 2.10.1 Antibody specificity and affinity

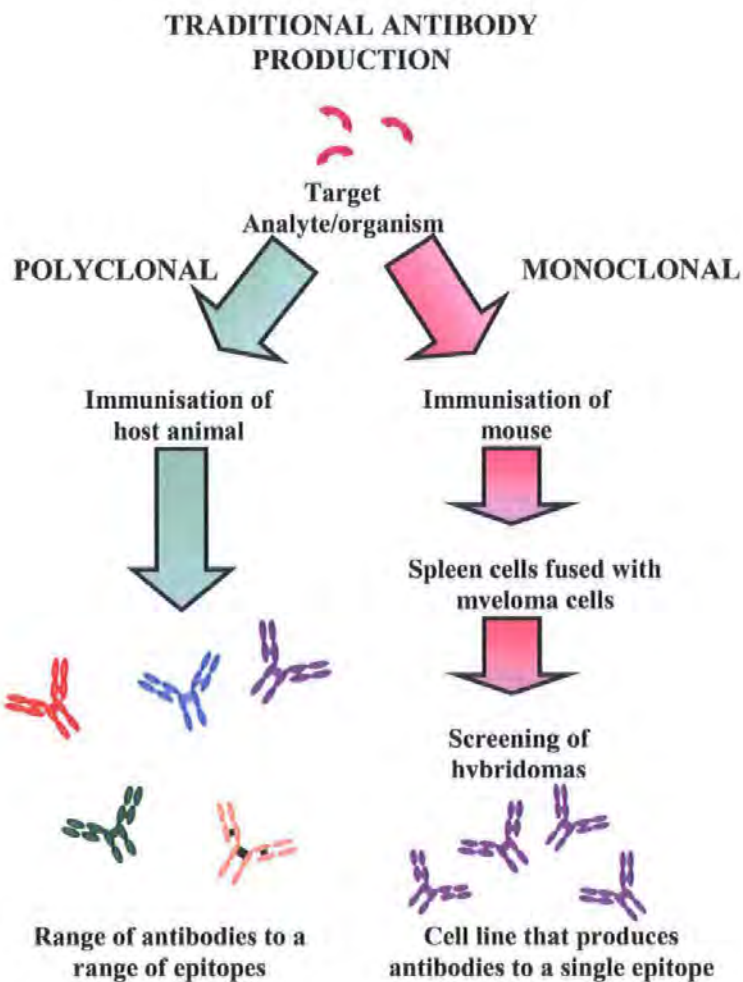
Each of the CDRs folds independently and the high level of variability in primary structure is responsible for the observed specific (or idiotypic) immune response. The CDR-H3 region is thought to show the most significant differences in structure and length and consequently contributes the most to specificity (Padlan and Kabat, 1988; Wang *et al.*,

1991; Hoogenboom and Winter, 1992; Nissim *et al.*, 1994; MacCallum *et al.*, 1996; Morea *et al.*, 1997, 1998; Sgirai *et al.*, 1998; Oliva *et al.*, 1998; McCarthy and Hill, 2001). The areas within the hypervariable region that are directly in contact with the antigen contain the amino acids which demonstrate the most variability and are often referred to as the specificity determining residues (SDRs). The amino acid sequence within the CDRs, which is not involved in antigen binding or the loops that are formed, is designated the framework region (FR) (Padlan *et al.*, 1995). Although these regions are not directly involved in antigen recognition (Sheriff *et al.*, 1987; Wedemeyer *et al.*, 1997), it is thought that they probably play a crucial role in the steric position of the CDR. Therefore, they can have an indirect effect on antibody specificity and affinity by providing a scaffold for the CDRs (Lamminmäki *et al.*, 1999; Daugherty *et al.*, 2000; Kusharyoto *et al.*, 2002) or effecting tertiary structure (de Haard *et al.*, 1998). For example, decreases in antibody affinity due to single aa changes within the FR have been observed (de Haard *et al.*, 1998; Caldas *et al.*, 2003). This is supported by the study of humanised murine antibodies which demonstrated a reduction in affinity when CDRs were inserted into a human FR (Foote and Winter, 1992; Saldanha *et al.*, 1999; Caldas *et al.*, 2003).

Over the years, conflicting evidence has emerged as to how the CDRs evolve to dictate the specificity and affinity of a given antibody (Jerne, 1955; Ehrlich, 1897). However, Burnet's theory of clonal selection is widely accepted today (Burnet, 1957). Later work showed that V (variable), D (diversity, only found in the heavy chain) and J (joining) gene fragments were randomly assembled during the development of the B-lymphocyte (Bernard *et al.*, 1978; Brack *et al.*, 1978). More recently it has been suggested that the V(D)J recombination can occur after the development phase, in mature cells and this occurs in combination with hypersomatic mutation (Rajewsky 1998; Hertz *et al.*, 1998). This is how B-lymphocytes are capable of producing high affinity antibodies along with a general population effect whereby antigen receptors of B-lymphocytes acquire mutations that enhance antibody affinity and these cells are selected over those that have a lower affinity for the antigen (Diaz and Casali, 2002). It has been suggested that during somatic mutation changes are made to the SDRs and surrounding amino acids, thought to be involved in stabilisation of the SDRs to allow for the optimal conformation for binding to a given antigen (Wedemeyer *et al.*, 1997). The exact molecular mechanisms for hypermutation within immunoglobulin genes are poorly understood and will not be discussed in this review.

### 2.10.2 Traditional methods for antibody production

The production of poly and monoclonal antibodies is summarised in Figure 2.8. There are particular disadvantages to the traditional approach to polyclonal and monoclonal antibody production. The production of antibody is reliant on a mammalian immune response, and therefore it is difficult to produce antibodies to low molecular weight analytes, non-immunogenic targets that are similar or identical to 'self'-molecules, such as sugars, toxic substances, unique epitopes, rare epitopes or those that are non-immunodominant that could be targets for detection assays (Emanuel *et al.*, 2000).



**Figure 2.8 Traditional antibody production.** An overview of the production of polyclonal and monoclonal antibodies is shown.

### *2.10.2.1 Polyclonal antibodies*

In the case of a polyclonal antibody, the antibody will recognise a range of undefined antigens and epitopes, frequently resulting in cross reactivity and, only a small proportion of the antibodies may react against the target. Furthermore, the performance and characteristics of an antibody produced from animal sera may vary even between the same species. As a consequence, the production of reagents in a reproducible manner is often difficult. In the case of polyclonal antibodies, it has been suggested that a specific target can be identified and antibodies produced, through the use of affinity purification (Petrenko and Volyanoy, 2003). However, this requires knowledge of suitable targets and expression of the recombinant protein in the correct conformation. Additionally, it is an expensive and time consuming procedure, particularly for large scale production.

### *2.10.2.2 Monoclonal antibodies*

In the case of a monoclonal antibody only one epitope, often of an unknown protein, is recognised. The use of detection reagents against unidentified and uncharacterised proteins could cause significant limitations to a detection assay. In the case of monoclonal antibodies, variation should be minimal due to the antibody resulting from the fusion of a B lymphocyte with an immortalised cell line. In monoclonal antibody production, extensive screening (usually by ELISA) is required to select cell lines with the desired properties, usually specificity and sensitivity. These cell lines then have to be propagated and maintained (Iqbal *et al.*, 2000). Therefore, antibodies that are produced in a mammal may not be the ideal way to produce specific detection reagents. A review of the advantages and disadvantages of poly- and monoclonal antibodies within detection immuno-assays has been conducted by Andreotti *et al.* (2003) and are summarised with the advantages and disadvantages of the use of engineered antibodies in Table 2.2

## **2.11 Engineered Antibodies**

Due to the inherent limitations of natural antibodies, particularly with regards to their use within biosensors (as discussed in section 2.9) alternatives to these natural molecules have been sought, and one avenue of research has been the production and use of engineered antibodies.



### 2.11.1 Chemically engineered antibodies

The use of antibody fragments, rather than the entire antibody molecule can be advantageous for a range of applications. Enzymatic digests of IgG molecules can be achieved by using papain to yield Fab or by using pepsin to produce a 100 kDa  $F(ab')_2$  fragment, Fab' fragments can be produced through the reduction of  $F(ab')_2$  fragments at the heavy chain disulphide groups using a reducing agent, commonly 2-mercaptoethylamine (Figure 2.9). The number of free sulfhydryl groups is dependent on both the species from which the antibody was derived and on the immunoglobulin subclass. One advantage of the exposure of free sulfhydryl groups is that they can be used for site specific attachment to a solid support. Enzymes such as trypsin or ficin can also be used as they cleave a range of immunoglobulins in the hinge region of heavy chain pairs (Josić and Lim, 2001).

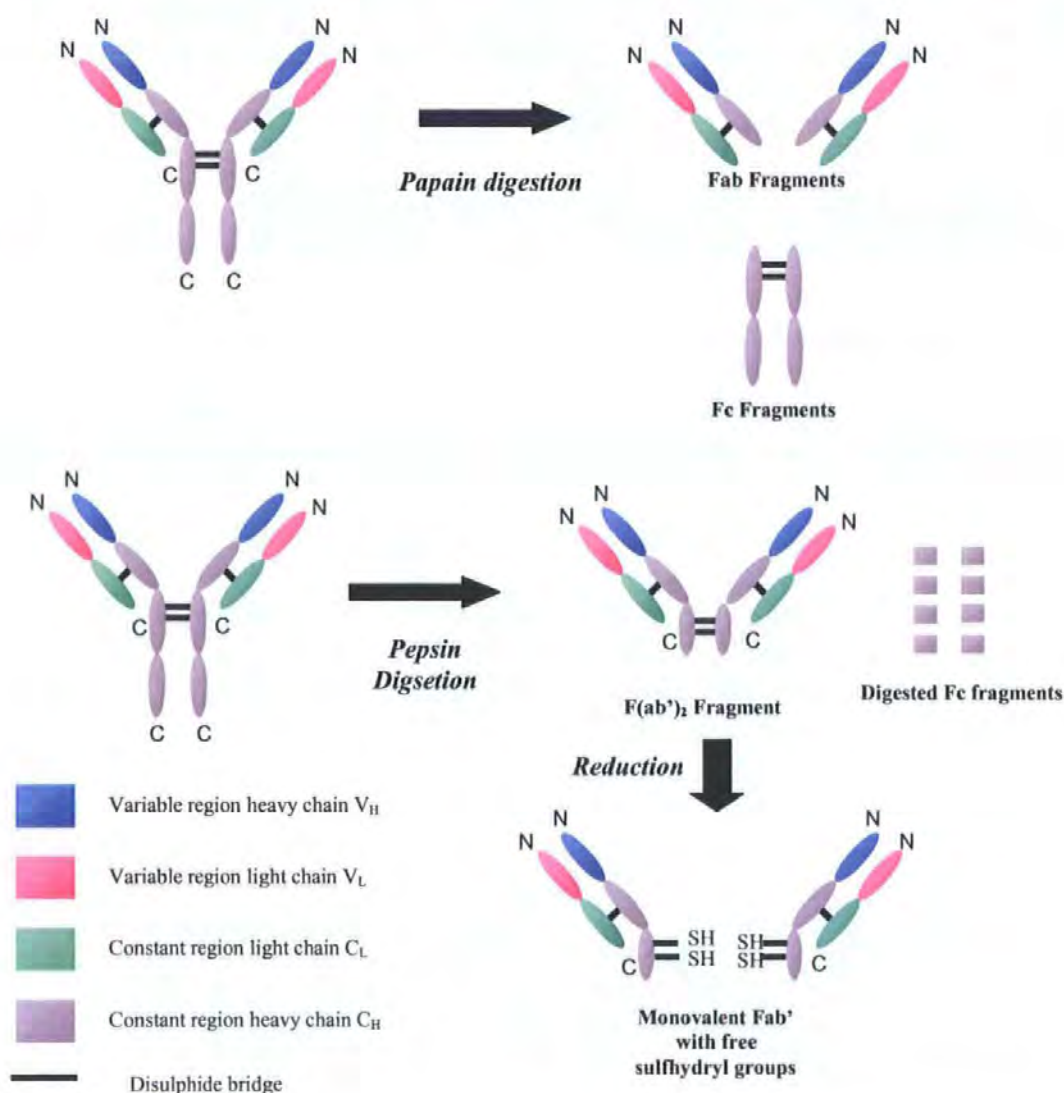


Figure 2.9 Digestion of IgG to produce Fab,  $F(ab')_2$  and Fab'

The use of Fab fragments has distinct advantages over the parent antibody both for *in vivo* and *in vitro* applications (Subramanian and Velander, 1996; Hudson, 1999). Conjugation of different antibody fragments can also be used to produce antibodies that are specific for different targets (Martin *et al.*, 1997). In the context of detection assays, the increase in sensitivity often observed is thought to be due to the increased density of binding sites and the ability for site specific attachment to certain solid supports exposing all antigen binding regions (Itoh *et al.*, 1999). Furthermore, the use of Fabs may be more applicable to certain biosensor technologies. For example, for ion channel biosensors the use of Fab' fragments is required as they can be correctly orientated and have only one recognition site for the target antigen, thus ensuring 1:1 binding. They can also be immobilised to a higher density again increasing the sensitivity of the assay (Cornell *et al.*, 1997). However, disadvantages of the use of Fab fragments detailed in Table 2.2 make production expensive (Cheung *et al.*, 2003; Jones and Landon, 2003). Loss of binding activity during digestion or purification, as well as the inherent constraints imposed by use of the parent antibodies produced by traditional methods (Table 2.2) also limit the applicability of these engineered antibodies.

### **2.11.2 Genetically engineered antibodies**

With the advances in recombinant DNA technology, it may be possible to tailor a new class of genetically engineered antibodies to suit the requirements of the detector and assay. Recombinant antibody technology combines phage display and cDNA libraries to allow for the production of a large number of immunoglobulin (Ig) fragment clones. Fab fragments can also be produced using recombinant techniques (Better *et al.*, 1988); however, smaller recognition elements incorporating the variable domains i.e. the sites required for antigen recognition have also been produced. The variable regions can either be connected by: (a) non-covalent forces to produce Fv (Skerra and Plückthun, 1988); (b) covalently using linkers such as disulphide bridges to create dsFv (Glockshuber *et al.*, 1990); or (c) a peptide linker used to produce a scFv (Bird *et al.*, 1988; Huston *et al.*, 1998). The following sections discuss the use of scFv. The first step in the production of scFv is the construction of a suitable gene library, which creates a source of recognition elements with differing binding specificities. Once a library has been produced, it can be screened for scFv that bind the target of interest. Consecutive rounds of selection allow for enrichment of the population for those bacteriophage phage expressing scFv that bind to the target of interest (Alfthan, 1998). The scFv DNA sequence can be examined and used to determine any features that may be important for binding that could be used for further

genetic manipulation. One of the distinct advantages of the use of recombinant antibodies is that they can be modified for successful incorporation into biosensors. For example, the affinity or specificity of a selected scFv can be enhanced using genetic manipulation or through experimental design (Griffiths *et al.*, 1994; Krebs *et al.*, 2001). Furthermore, protein engineering techniques can also be used to tailor the properties of the antibody for the required purpose, including the incorporation of specific immobilisation tags (Piervincenzi *et al.*, 1998). Protein engineering has also been investigated as a method of changing the signal transduction properties of the binding molecule (Hellings and Marvin, 1998). The ability to incorporate signal transduction into a recognition element is highly desirable as it allows for simplification of biosensor design and a reduction in the use of reagents (Hellings and Marvin, 1998). A number of approaches can be utilised including incorporation of a fluorescent moiety that is sensitive to changes in the environment, such as those induced by antigen binding, producing a measurable variance in signal (North, 1985; Marvin *et al.*, 1997; Marvin and Hellings, 1998; Renard *et al.*, 2002, 2003; Jespers *et al.*, 2004). Alternatively fluorescence resonant energy transfer (FRET) can be used to report analyte binding (Förster, 1959; Stryer and Haugland, 1967; de Silva *et al.*, 1997; Selvin 2000). However, successful use of this approach is generally limited to recognition elements that show a large intrinsic change in conformation on analyte binding such as aptamers (Jhaveri *et al.*, 2000) or the use of a more complex engineering strategies, in the case of recombinant antibodies (Medintz *et al.* 2005). This extremely elegant approach could not have been achieved through the use of more traditional recognition elements such as poly or monoclonal antibodies and represents a very exciting development in the use of recombinant antibodies as reagents on biosensor platforms.

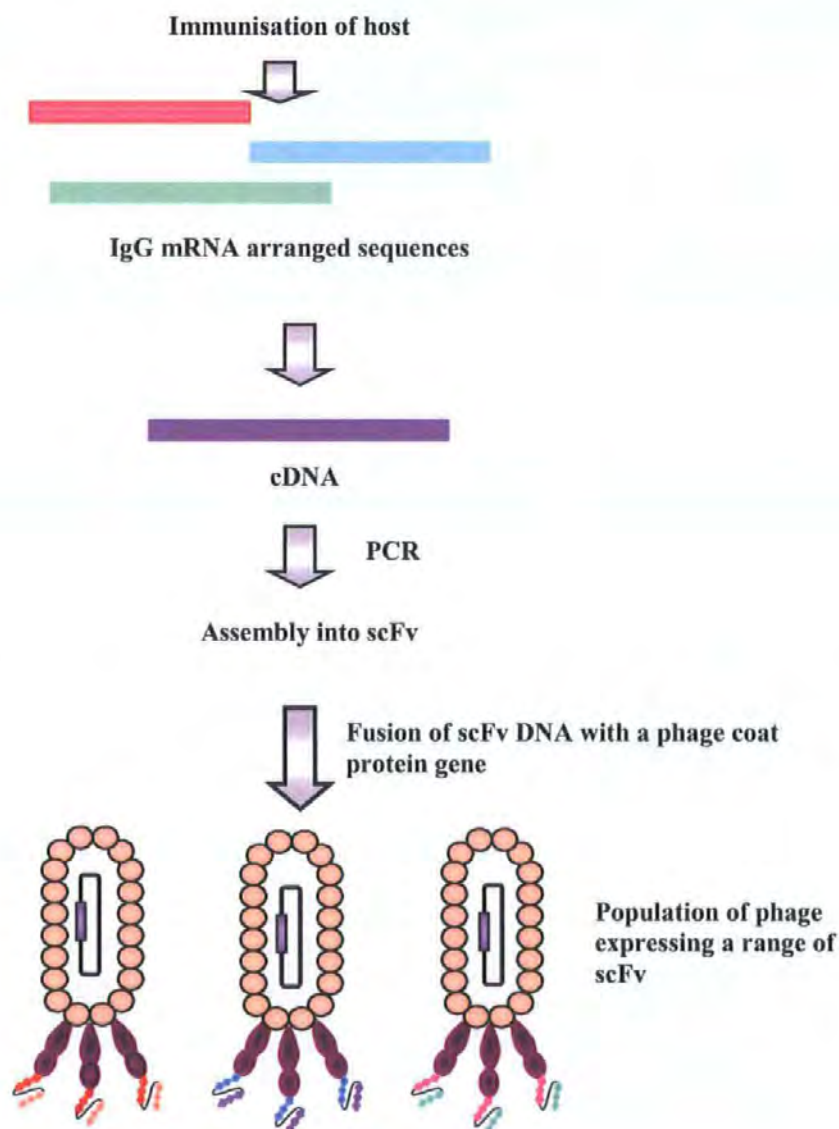
#### **2.11.2.1 Production of genetically engineered antibodies**

Generation of recombinant antibodies is commonly undertaken by the use of phage display that can either be produced from a monoclonal cell line, naïve, synthetic or immunised libraries (Willats, 2002). Recombinant forms of monoclonal antibodies with high affinity and specificity can be produced; this is particularly advantageous if the cell line is unstable or for engineering specific attributes, such as immobilisation tags. This technique can be hampered by the presence of aberrant mRNA sequences within the hybridoma resulting in the generation of antibody fragments that do not have the same attributes as the parent antibody (Duan and Pomerantz, 1994; Ostermeier and Hill, 1996). This can, however, be overcome by the use of recombined V<sub>L</sub> and V<sub>H</sub> sequences as described by Krebber *et al.* (1997). Synthetic libraries are constructed from V-gene sequences with modifications at



particular sites. However, for these libraries to be successfully employed knowledge of the CDRs is required and can also be utilised to construct libraries for defined epitopes (Hoogenboom and Winter, 1992; Kirkham *et al.*, 1999). In the case of a naïve library, the antibodies originated in an animal that was not deliberately challenged with a potential target. These libraries have been used successfully for the generation of recognition elements against a range of target analytes (Winter *et al.*, 1994; Haardt *et al.*, 1999). A distinct advantage of the use of naïve libraries is that, as with synthetic recognition elements, production of recognition elements against toxic or non-immunogenic markers is possible (Griffiths *et al.*, 1993; Nissim *et al.*, 1994). However, these libraries may be limited by diversity and it has also been observed that the size of the library is directly proportional to the affinity of the antibodies isolated (Griffiths *et al.*, 1994). In the case of an immunised library, the animal was exposed to a target of interest and as a result will have a large proportion of antibodies directed against the target and a degree of affinity maturation will have been conducted *in vivo* (Clackson *et al.*, 1991). Thus, the use of these libraries has frequently resulted in the isolation of higher affinity antibodies than those selected from a naïve source (Clackson *et al.*, 1991). There are also limitations of the production of antibodies when using immune libraries. For example these libraries only allow for the isolation of scFv against antigenic and/or non-toxic substances and the library may be biased towards immunodominant and abundant epitopes (Hoogenboom *et al.*, 1998). In the case of immune libraries, antibody sequences are usually obtained from the B-cells originating from the spleen of the immunised host (Willats, 2002). Initially, the immunoglobulin V domains from the naïve or immunised source are amplified by PCR. Kirkham and Shroeder (1994) suggested that each of the segments of this domain were important in forming the structure of the actual binding site. Furthermore, V genes have been implicated as being heavily involved in specific immune responses and it is the V genes that are utilised in the production of engineered antibodies. Antibody fragment genes have been successfully inserted into a wide range of vectors to facilitate surface display; including bacteria, viruses, bacteriophage and yeast (McCafferty *et al.*, 1990; Clarkson *et al.*, 1991, Fuch *et al.*, 1991; Marks *et al.*, 1991; Hoogenboom *et al.*, 1991; Kieke *et al.*, 1997; Motterhead *et al.*, 2000). One of the most commonly employed methods is the use of bacteriophage primarily the filamentous bacteriophage M13. Expression of the scFv on the bacteriophage surface is usually achieved by creating a fusion protein with the N-terminus of the M13 coat protein, gp3 (Iqbal *et al.*, 2000), although other bacteriophage, phagemids and genes have also been employed for phage display of engineered antibodies, as reviewed by Benhar (2001).

The recombinant antibodies are then expressed on the surface of the phage as the antibody-encoding DNA is contained within the phage (Figure 2.10.). The advantage of the use of phage display for antibody engineering is the close link between genotype and phenotype. Either gene libraries or hybridoma cells can be used to produce recombinant antibodies by bacteriophage display, although more recent work has applied ribosome display in which the scFv are transcribed and translated in a cell free system (Hanes *et al.*, 2000). The complexes can then be used to bind to a chosen target using a procedure often termed biopanning (as discussed in section 2.11.2.2), which continues as with phage display. Using this method, and with the incorporation of RT PCR with a low fidelity polymerase, Hanes *et al.* (2000) produced scFv from a naïve synthetic library that had affinities within the picomolar range.

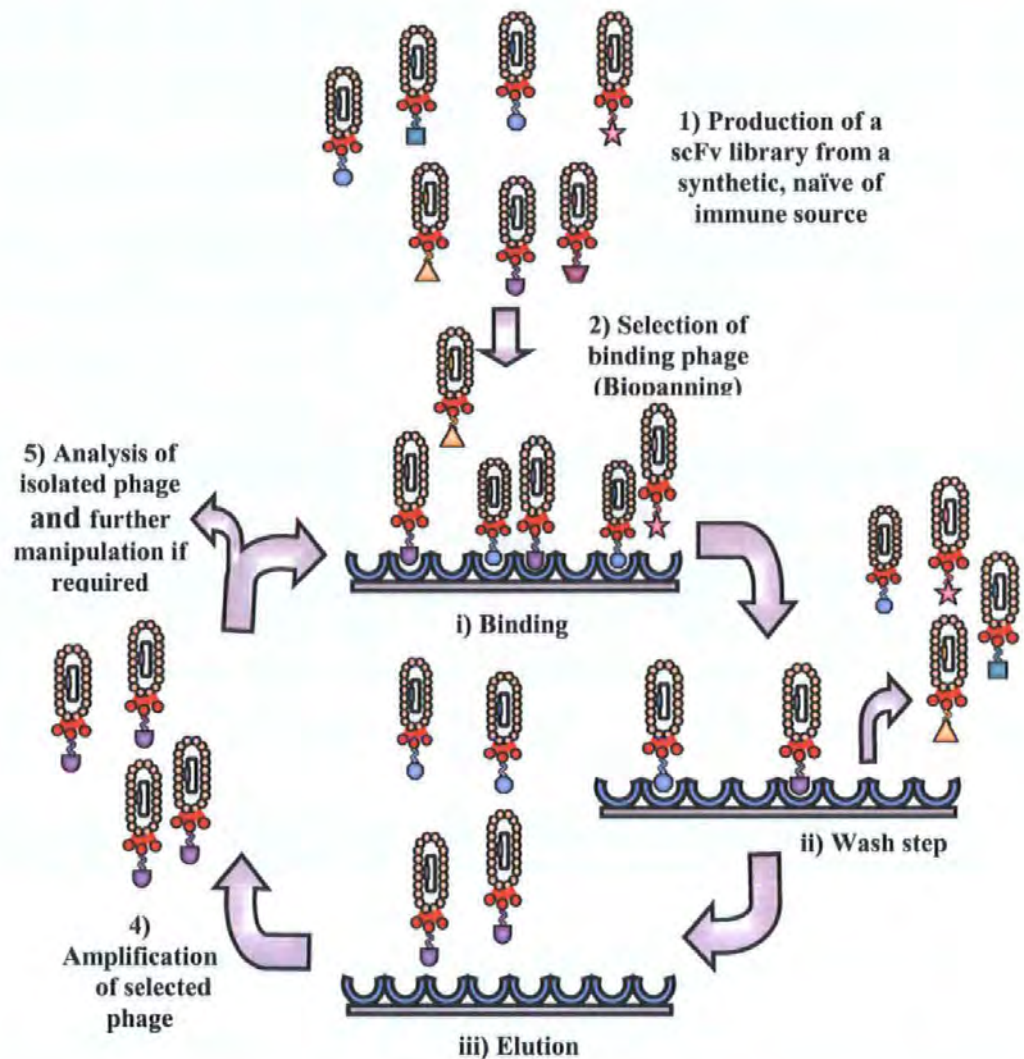


**Figure 2.10** A schematic representation of an example of bacteriophage display using an immunised library.

This method relies on the fusion of millions of antibody encoding genes to the coat protein genes of a bacteriophage. Following immunisation, mRNA is isolated, reverse transcription of the RNA allows construction of the scFv library. PCR amplification of antibody sequences, overlap extension PCR followed by cloning of the assembled scFv sequence into a suitable vector allows for expression of the fusion construct on the surface of each phage as the coat protein also encoding the scFv gene is incorporated into the phage particle. The resultant library contains a large number of phage each displaying different scFv.

#### **2.11.2.2 *Biopanning***

Once a library has been produced, it can be screened for scFv that bind the target of interest. Usually antigen is immobilised onto a surface, for example by passive adsorption or by biotinylation with subsequent binding onto a streptavidin coated surface (Willats, 2002). Unbound phage are washed away, leaving only those that bind to the target. Finally, the scFv are eluted from the target and propagated using a suitable bacterial host. Consecutive rounds of selection allow for enrichment of the population for those phage expressing scFv that bind to the target of interest (Figure 2.11), a procedure often termed 'biopanning' (Alfthan, 1998).



**Figure 2.11 Schematic representation of a possible biopanning procedure for scFv against a particular target.** (1) Production of a scFv library (2) Immobilisation of the target of interest onto a solid support and subsequent addition of a representative proportion of the scFv (ii) Those phage displaying scFv that recognise the target analyte are bound (iii) Unbound phage are removed by a series of wash steps (iv) Bound phage are eluted from the target analyte (4) The eluted phage that recognise the target analyte are used to re-infect a suitable bacterial host to allow for amplification of phage displaying scFv specific to the target analyte. At this stage the cycle can be repeated or (5) The phage can be evaluated for the desired characteristics, characterised and manipulated further if required.

### **2.11.2.3 Enhancing the Specificity of scFv**

Although scFv can be manipulated to enhance specificity, there are problems associated with using surface exposed antigens as targets. As with any other means of recognition element production, purified or recombinant antigen may not be available. Furthermore, any purification strategy utilised may denature or alter the antigen of interest. If recombinant protein is used, the protein structure may differ from the native form or from that found when anchored to a cell surface. Consequently, there may be an observed decrease in the binding properties of the scFv when the antigen is presented on the surface of a whole cell or spore. One method of producing antibodies against a given target is through the production of an epitope mimic, also referred to as an anti-idiotypic antibody (Hombach *et al.*, 1998). The use of anti-idiotypic antibodies has had varying degrees of success. Often, increased binding to the mimic compared to the native target has been observed (Goldbaum *et al.*, 1997). Other important techniques for enhancing specificity that have been reported are competitive panning and competitive elution. These have provided a means to select epitopes that are not shared by two related antigens. Krebs *et al.* (2001) described a method by which pre- and post- adsorption steps could be utilised to select out those scFv that bound to possibly cross reactive targets to produce specific scFv. As an alternative to this method, Scherer *et al.* (1998) described a methodology by which polyclonal antisera were produced against a specific target and the sera were depleted of antibodies that were not specific to the target by purification with the recombinant protein. The purified sera that recognised the antigen of interest were then used to construct a scFv library.

### **2.11.2.4 Enhancing the affinity of scFv**

Genetically engineered antibodies produced from an immune library generally have higher affinities than antibodies derived from a naïve source (Griffiths *et al.*, 1994). However, it is possible to manipulate the fragments by the use of further techniques to improve these affinities. Techniques such as random mutagenesis (Ferrer *et al.*, 1999; DeHaard *et al.*, 1999; Ridgeway *et al.*, 1999; Beiboer *et al.*, 2000) or site-directed mutagenesis (Morrow, 2000) have been used. However, there are inherent problems associated with both approaches. In the case of random mutagenesis; as the mutations are non-specific, they often give rise to undesirable properties particularly when using an expression vector (Moore and Maranas, 2004). In the case of site-directed mutagenesis, lack of knowledge, of the structure-function relationship can present problems with mutated scFv with no



ability to bind to the target (Morrow, 2000). Other methods of enhancing affinity include ribosomal display and chain shuffling (Jackson *et al.*, 1992; Kirkham *et al.*, 1994; Alftan, 1998). The use of *in vitro* methods to enhance the affinity of a library has achieved affinities in the nano to picomolar range (Rader and Barbas, 1997; Alftan, 1998). Other ways of enhancing the affinity of a recognition element to a protein can be drawn from techniques developed in other areas. For example, the affinities of synthetic recognition elements have been enhanced through the use of lower concentrations of target in a specific selection procedure. Mutagenesis, to improve scFv specificity and affinity, is based on the determination of hypervariable regions and the analysis of mutations within the V genes. Increasing knowledge in this area would lead to a better understanding of fundamental mechanisms that could then be applied to phage display or, more likely, to *in vitro* systems such as ribosomal display.

#### 2.11.2.5 *Enhancing stability and engineering for correct orientation*

Protein engineering techniques can also be used to tailor the properties of the antibody for the required purpose, such as enhanced stability or use of fusion protein to engineer specific tags for correct orientation. Many applications for detection technologies require that a recognition element is stable within harsh environmental conditions. However, natural antibodies have demonstrated differing stabilities and it has been put forward that even single amino acid changes can affect antibody stability (Honegger and Plückthun, 2001; Ewert *et al.*, 2003). Ways of increasing antibody stability have included; (a) CDR grafting into frameworks of known tolerance to the conditions required, (b) the introduction of mutations known to enhance stability, or (c) the use of selective pressure (i.e. use of the conditions required) within the selection process (Jung *et al.*, 1999; Ewert *et al.*, 2004). To allow for correct orientation of a recognition element on a solid support, such as that used on a biosensor, fusion proteins are the common method of choice. Commonly histidine tags (His-Tags) are fused into scFv when expressed in its soluble form and this is then utilised as a method for purification, detection or sometimes immobilisation of the scFv (Lindner *et al.*, 1997). The use of an unpaired cysteine at the C-terminus to allow for covalent attachment has also been reported (Kipriyanov and Little, 1997). Specific modifications at the C-terminus have the advantage of maintaining 1:1 ratio of recognition element to immobilisation group, ensuring that the binding site is exposed (Piervincenzi *et al.*, 1998). Modifications such as this can be used to enhance scFv

properties. Once the appropriate clone has been selected or engineered, it is usually required that it is expressed in a soluble form.

#### **2.11.2.6 Expression of scFv in a soluble form**

The isolation of scFvs from a phage display or ribosomal display library has been well documented; however, complications can arise when trying to express the scFv in a soluble form. When scFvs are cloned into bacterial expression vectors the yield is often poor. This can be due to a number of reasons; most commonly the scFv is not contained within the periplasm and may aggregate and precipitate. Alternatively, cytoplasmic inclusion bodies may form containing scFvs with either reduced or no binding activity. The ease with which a scFv can be expressed in a soluble form and undergo correct folding varies with each individual scFv. In general, it has been found that those scFv produced by phage display refold and solubilise more readily than those produced from hybridoma cell lines. This is likely to result from the method used in phage display, whereby those scFv that possess the required binding characteristics and are expressed well are selected and possibly over-represented within a system. By contrast, those scFv that may bind well, but are toxic to the cell, or those which are relatively rare and possess unusual characteristics may not be selected. For this reason, it may be necessary to investigate a range of expression vectors and refolding and solubilisation strategies. Some that have already been investigated include yeast and insect expression systems (Mahiuoz *et al.*, 1998).

Advances in recombinant DNA technology and computational molecular biology have allowed the production of alternatives to traditional antibodies that can be modified to suit the requirements of the detector and assay design (Irving *et al.*; 1996; Lindner *et al.*; 1997; Piervincenzi *et al.*, 1998; Jung *et al.*, 1999). The advantages in the use and production of recombinant antibodies compared to traditional approaches are summarised in Table 2.2.

	<b>Polyclonal</b>	<b>Monoclonal</b>	<b>Chemically engineered antibodies</b>	<b>Genetically engineered antibodies</b>
Animal use	Yes – required each time	Initial – mouse immunised, further production of antibody from cell line	As for parent polyclonal or monoclonal antibody	Recombinant DNA technology may require initial use, but not always necessary
Affinity and specificity	Variable within a batch and from animal to animal	Reproducible - higher specificity, single epitope, affinity dependent on antibody	Specificity can be altered using conjugates of antibody fragments	Reproducible - high specificity and affinity
Targets	Variable within a batch and from animal to animal	Single target, difficult to isolate specific clones that recognise a unique epitope	As for parent polyclonal or monoclonal antibody	Can be manipulated to recognise unique, rare, sterically shielded or non-immunodominant epitopes
Tailoring of antibody to suit requirements	No	No	Limited	Yes
Supply	Finite supply of an antibody from sera	Infinite supply from immortalised cell line	As for parent polyclonal or monoclonal antibody	Infinite supply from mammalian, or more commonly, bacterial cells
Logistics	Use of animals and ongoing effort to produce quality anti-sera to a wide range of agents	Expensive and time consuming to maintain	As for parent polyclonal or monoclonal antibody, also requires digestion and further purification	Stable genetic source expression and production (bacterial fermentation) less expensive and less time consuming

**Table 2.2** A comparison of traditional chemically engineered and genetically engineered antibodies.



## 2.12 Summary

Sensitive and specific detection of BW agents is essential in a military context for the protection of armed forces and the maintenance of an effective military response and in a bioterrorism scenario for the protection and effective treatment of a susceptible civilian population. In both cases, the timely administration of appropriate treatment following exposure and effective decontamination of affected areas is critical, making rapid detection of these pathogenic microorganisms essential.

Bacterial endospores of *B. anthracis* represent the most significant form of the organism in a BW scenario due to their resistance to extreme environmental conditions, ability to survive for prolonged time periods, ease of production, effective dissemination and their ability to infect humans, particularly via the respiratory route. Rapid real time detection of *B. anthracis* is a particular problem and has been stated as 'not yet technologically practical' (Webb, 2003). Systems deployed in the US to detect airborne anthrax were reported to have problems with false positives and (of more concern) false negatives and had a time lag between dissemination and identification of 12-24 h (Webb, 2003). A number of methods are available for detection of *B. anthracis*; however, only a small proportion of these could be utilised in the field, and of these, only a minority are capable of true real time specific detection. Of these, optical biosensors have shown particular promise; however, the detection of larger particulates has been problematic often leading to a significant reduction in assay sensitivity. Many detection assays, including those employed by optical biosensors, utilise the detection of a binding event, most commonly antigen to antibody in the detection of aerosolised BW agents. However, lack of sensitivity and specificity is often observed. Therefore, increased knowledge of the structural and antigenic components of the *B. anthracis* spore and the provision of suitable recognition elements is essential for the specific detection of this BW agent. This work aims to overcome these challenges and to elucidate structural components that may be utilised for the production of high affinity specific recognition elements to *B. anthracis* spores.

## Chapter 3 Materials and methods

### 3.1 Bacterial strains and plasmids

Initial investigations of sonication and surface labelling techniques were undertaken using a non-pathogenic organism as a model system. Stock suspensions of *Bacillus atrophaeus* spores were obtained from the Health Protection Agency (HPA) UK. *Bacillus atrophaeus* has been re-classified and was previously *B. subtilis* var. *niger*, previously *B. globigii* (*B. atrophaeus*).

For the evaluation of *B. anthracis* spores a double cured strain (UM23Cl<sub>2</sub>, pXO1<sup>-</sup>, pXO2<sup>-</sup>) was used (kindly provided by Dr. C. Thorne). Strain RBA91 (pXO1<sup>-</sup>/pXO2<sup>-</sup>/Sap<sup>-</sup>) and strain SM91 (pXO1<sup>-</sup>/pXO2<sup>-</sup>/Eag<sup>-</sup>) were kindly provided by the Pasteur Institute. The wild type Ames strain (pXO1<sup>+</sup>/pXO2<sup>+</sup>) of *B. anthracis* acquired from stocks held at Dstl was also used for this study. For evaluation of antibodies,  $\gamma$ -irradiated wildtype Ames, kindly provided by the Health Protection Agency, UK and Sterne (pXO1<sup>+</sup>/pXO2<sup>-</sup>), strain kindly provided by Defence Science Technology Organisation (DSTO), Australia, spores were used.

*B. cereus* (NCTC 11143, NCTC 9946 and NCTC 11145), *B. pumilus* (NCTC 10337), *B. brevis* (NCTC 2611), *B. coagulans* (NCTC 10334), *B. atrophaeus* (NCTC 10073), *B. thuringiensis* var. *kurstaki* and *B. thuringiensis* var. *israelensis* were obtained from NCTC (Public Health Laboratory National Collection of Typed Cultures).

### 3.2 Preparation of *Bacillus* spores

A single colony was inoculated into 50 ml of LB Broth (10 g/l Difco Bacto tryptone, 5 g/l Difco Bacto Yeast extract, 10 g/l NaCl, Difco Bacto agar adjusted to 11 in distilled water) in a 250ml conical flask and incubated for approximately 6 h at 37°C. Vent capped 150 cm<sup>2</sup> tissue culture flasks containing 75 ml of new sporulation medium (25 g/l Difco Bacto Agar, 3 g/l Oxoid Tryptone, 6 g/l Oxoid Bacteriological peptone, 3 g/l yeast extract (Oxoid), 1.5 g/l Lab Lemco powder 1 ml/l of a 0.1% solution MnCl<sub>2</sub>.4H<sub>2</sub>O) for *B. anthracis* spores or isolation agar for all other *Bacillus* species (6 g/l Oxoid nutrient broth No. 2, CM67, 0.3 g/l manganese sulphate (MnSO<sub>4</sub>.H<sub>2</sub>O, 0.25 g/l KH<sub>2</sub>PO<sub>4</sub>, 12.0 g/l Oxoid technical agar No. 3, L13) were inoculated with 2.5 ml to 5 ml of the 6 h culture and incubated at 37°C until the culture contained at least 95% phase bright spores, determined

by phase contrast microscopy. The spores were recovered by resuspension in 15 ml of ice-cold sterile distilled water and were harvested by centrifugation at 10000 g for 10 min at 4°C. The supernatant was removed and the pellet resuspended in ice-cold sterile distilled water.

### **3.3 Washing spores**

Spores were centrifuged at 10000 g for 10 min at 4°C, the supernatant was discarded and the pellet resuspended in ice-cold sterile distilled water. This was repeated as many times as required to achieve > 95% phase bright spores (usually 10). After the final wash the spore pellet was resuspended in approximately 40 ml of sterile ice-cold sterile distilled water per 200 ml of start volume. A serial 10 fold dilution of washed spores was plated onto LB agar plates (10 g/l Difco Bacto tryptone, 5 g/l Difco Bacto Yeast extract, 10 g/l NaCl, 1.5% (w/v) Difco Bacto agar to 1 l in distilled water) in triplicate (250 µl per plate). The remaining diluted samples were incubated at 60°C for 1 h to lyse the vegetative cells, these were again plated on onto LB agar plates (in triplicate (250 µl per plate). All plates were incubated overnight at 37°C. The number of spores and vegetative cells in each sample was calculated. The presence of >95% phase bright spores was confirmed using phase contrast microscopy. Spore suspensions, at approximately  $1 \times 10^9$  cfu/ml were stored in sterile distilled water at 4°C or -20°C.

### **3.4 Urografin purification of *B. anthracis* spores**

Urografin purification has been used previously to highly purify spores (Williams and Turnbough, 2004). *B. anthracis* UM23C12 spores that had been washed ten times in ice-cold sterile distilled water were subsequently Urografin purified and washed a further three times in ice-cold sterile distilled water, as described by Williams and Turnbough (2004).

### **3.5 Wildtype Ames exosporium preparation**

Unwashed wildtype Ames exosporium was kindly provided by Dr. C. Redmond (Dstl); the growth of the strain, preparation of spores and exosporium purification and removal were all conducted as described previously (Redmond *et al.*, 2004).

### 3.6 FITC labelling of spores

Known concentrations, 1 mg/ml, 0.5 mg/ml and 0.2 and 5mg/ml, of FITC (fluorescein isothiocyanate isomer I, Sigma) were made in 0.1 M sodium bicarbonate buffer pH 9 (Sigma) immediately prior to use. Washed *B. atrophaeus* spores were diluted to  $1 \times 10^9$  spores/ml in 0.1 M sodium carbonate bicarbonate buffer pH 9 (Sigma). The FITC was then added as detailed in Table 3.1. The FITC spore mix was covered in aluminium foil and left to incubate for 2 h at room temperature on a DYNAL windmill to provide continuous mixing. The FITC spore mix was then centrifuged at 10000 g and resuspended in PBS (10 mM sodium phosphate buffer pH 7.4 138 mM KCl, 27 mM NaCl, Sigma), this was repeated three times. The FITC labelled spores were then viewed using fluorescence microscopy, to determine the optimum labelling protocol.

Final amount of FITC to be added	Spores @ $1 \times 10^9$ spores/ml	FITC stock concentration (mg/ml)	Volume of FITC stock to be added ( $\mu$ l)
200 $\mu$ g	1	1	200
100 $\mu$ g	1	1	100
50 $\mu$ g	1	1	50
25 $\mu$ g	1	0.5	50
12.5 $\mu$ g	1	0.25	50

**Table 3.1. Optimisation of FITC labelling detailing the volumes of FITC required for differing amounts to be added to 1ml of  $1 \times 10^9$  spores/ml.**

The optimal amount of FITC appeared to be 200  $\mu$ g, therefore the spores were labelled as described previously. The spores were then sonicated and centrifuged at 10000 g for 10 min and the supernatants removed. The proteins within the supernatants were separated by SDS PAGE. To visualise the bands on a gel two methods were investigated. One involved the transmission of blue light through the gel, the other just utilising the UV light on a Gel Documentation system (BioRAD). As a control FITC labelled antibody was also analysed. Once any FITC labelled protein bands had been identified the gels were photographed and stained using a Colloidal Blue stain (Invitrogen) according to the manufacturer's instructions. Supernatants were also concentrated using Vivaspin500 filters (5000 MWCO) and the analysis by SDS-PAGE (section 3.27).

### 3.7 Biotinylation of the spore surface

Washed spores were diluted to approximately  $1 \times 10^9$  cfu/ml and centrifuged (10000 g for 10 min). The supernatant was removed and replaced with 1 mg/ml sulfo-NHS biotin (Perbio) in PBS and incubated for various time periods on ice. The spores were then washed twice in PBS and once in 50 mM Tris pH 7.5, 50 mM magnesium chloride ( $\text{MgCl}_2$ ), to quench and remove excess biotin. When the biotinylation was complete the spores were centrifuged at 10000 g for 10 min and the supernatant removed and replaced with sterile distilled water (Invitrogen), this was repeated three times to ensure all excess biotin was removed. The spores were then sonicated (section 3.18.2) or electroporated (section 3.15), centrifuged at 10000 g for 10 min and the supernatants removed for analysis. To visualise the biotinylated proteins the supernatants from the sonicated spores were separated by SDS-PAGE on 4-12% Bis-Tris Novex gels (Invitrogen), as described in section 3.27 blotted onto PVDF membranes and probed using a streptavidin HRP conjugate (Sigma) 1: 1000 dilution in PBS Tween 20 0.1% for detection of the molecular weight marker (MagicMark™ Western Standard, Invitrogen), as described in section 3.28. To ensure the streptavidin peroxidase conjugate did not bind non-specifically to any proteins released during sonication, non-biotinylated spores were also sonicated and analysed by the same method described above. Once the procedure had been optimised various *Bacillus* species were analysed using this method and differences between the profiles examined.

### 3.8 Enrichment of biotinylated proteins

After biotinylation and sonication spores were centrifuged at 10000 g for 10 min and the supernatant removed and filtered using a 0.2  $\mu\text{m}$  low protein binding filter to remove any intact spores. To capture the biotinylated protein 1 ml aliquots of Magnabind streptavidin magnetic beads were used (Perbio ). The beads were washed three times in sterile phosphate buffered saline PBS (BupH pH 7.4, Perbio ) with the use of a MagnaBind Magnet (Perbio), to separate the magnetic particles from the liquid phase. After the last wash the PBS was removed and 1 ml of biotinylated proteins were added and incubated overnight at 2-8°C. Once the incubation was complete the beads were washed 5 times with sterile PBS and the biotinylated proteins eluted with 1 M KOH for 3 min with mixing. To neutralise the high pH from the KOH, 750  $\mu\text{l}$  fractions were eluted into 250  $\mu\text{l}$  1 M citric acid (BDH). The elution step was repeated three times and the fractions pooled, and the

elution buffer exchanged for PBS and the proteins concentrated using a Vivaspin500 column (5000 MWCO). Fractions were visualised by SDS PAGE using a 4-12% Bis-Tris NuPage gel (Invitrogen), SYPRO stained (Molecular Devices) according to the manufacturer's instructions. Samples were then analysed by nano-LC-tandem MS (section 3.31).

### **3.9 Preparation of S-layer proteins**

Bacterial cultures (50 ml) were grown overnight at 37 °C in SPY medium (60 mM K<sub>2</sub>HPO<sub>4</sub>, 45 mM KH<sub>2</sub>PO<sub>4</sub>, 15 mM ammonium sulphate, 10 mM magnesium sulphate, 2.4 mM sodium citrate, 0.2% (w/v) glucose 0.2% w/v yeast extract, all Sigma). Cultures were centrifuged at 8000 g for 30 min at 4 °C and resuspended in 5 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.2. The resuspended pellets were incubated for 2 h at 20°C with shaking, and then centrifuged at 6000 g for 10 min at 4 °C. The supernatant was removed and dialysed against 4 l of 50 mM Tris-HCl pH 7.5 overnight at 4 °C. S-layer self-assembly products were sedimented by centrifugation for 30 min at 4 °C. The precipitate and supernatant were analysed by SDS-PAGE to confirm the presence of S-layer proteins. The soluble S-layer protein contained in the supernatant was concentrated by ultrafiltration and filtered using a 0.45 µm filter. An aliquot of protein before and after concentration was retained for analysis. Further purification of S-layer proteins was achieved using a HiLoad 1660 Superdex 200 preparatory grade column (Amersham) and an AKTA FPLC system (Amersham), monitoring purity by SDS-PAGE (section 3.27). Fractions that contained pure S-layer protein were pooled and the concentration determined by BCA Assay (Pierce), according to the manufacturer's instructions.

### **3.10 Preparation of *Escherichia coli***

*E. coli* was taken from stock culture maintained at Dst1 and streaked out onto LB agar. A single colony was inoculated into 50 ml LB Broth and incubated for 6 h at 37°C. Stocks were enumerated by viable plate counts.

### **3.11 Preparation of ovalbumin**

A stock solution of Ovalbumin (Sigma, Grade V) was prepared at 10 mg/ml. Stocks were diluted down to the desired concentration.

### **3.12 Plasmids**

Plasmids pAK100 (used for phage display) and pAK300 or pAK400 (used for production of soluble scFv) were a kind gift from Dr A. Plückthun (University of Zürich, Switzerland), and were used as previously described (Krebber *et al.*, 1997). The pKAPPA plasmid was a kind gift prepared by Dr. C. Mayers (Dstl).

### **3.13 Antibodies**

For the detection of *B. anthracis* spores, a polyclonal rabbit anti-*B. atrophaeus* antibody (Dstl) was prepared using whole spores as the immunogen. For detection of *B. anthracis* spores a monoclonal or polyclonal anti- *B. anthracis* antibody was used (Dstl). Both were prepared using whole spore preparations of  $\gamma$ -irradiated wildtype Ames strain. For the identification of immunogenic proteins of the exosporium, a polyclonal antibody was used that had been prepared using washed wildtype Ames exosporium as the immunogen (Dstl). For the detection of ovalbumin a polyclonal rabbit anti-ovalbumin antibody was used (Dstl) prepared using ovalbumin (Sigma, Grade V) as the immunogen and for bovine serum albumin (BSA) detection a monoclonal anti-BSA antibody was obtained from Sigma. For the detection of the S-layer protein EA1 a monoclonal anti-EA1 antibody was used.

### **3.14 Antibody labelling**

Dimethyl formamide (1 ml) (DMF, Sigma) was added to the biotinylation or fluorescein reagent (Molecular Devices) using a 1 ml syringe. The solution was swirled gently to mix and then extracted from the vial. The appropriate amount of biotin or fluorescein, to obtain a specific molar coupling ratio (MCR), as detailed in the manufacturer's instructions, was then added to 0.5 or 1 ml of antibody at 1 mg/ml in sterile PBS and left to incubate at room

temperature in the dark for 2 h. The antibody was purified using a PD-10 column (Amersham), which was first washed with 25 ml of PBS (phosphate buffered saline), to allow the column to equilibrate. The antibody solution (0.5 or 1 ml) was loaded onto the column and 1.25 or 0.63 ml of PBS added. An additional 3 ml of PBS was loaded onto the top of the column and 0.5-1 ml fractions collected. The absorbance was read at 280 nm following purification using the PD-10 column, to determine the final antibody concentration at 362 nm to determine the amount of biotin present or at 490 nm to determine the amount of fluorescein present. The antibody concentration and the molar incorporation ratio (MIR) of biotin molecules per IgG was calculated according to the manufacturer's instructions.

### **3.15 Electroporation of spores**

Based on previous work, (Dr. M. McDonnell, unpublished data) samples were electroporated using a BTX 820R electroporator (Harvard Apparatus) using a square wave pulse. A 1 mm gap cuvette was used resulting in an applied voltage of 5 kV/cm between 1 and 99 0.1 s pulses or 7.5 kV/cm in 12 x 0.0001 s. Deionised water (18  $\Omega$ ohm) was used to minimise the possibility of arcing, especially at higher voltages. Electrodes in the disposable cuvettes were made from aluminium. An aliquot (100  $\mu$ l) of washed *B. atrophaeus* spores at an approximate concentration of  $1 \times 10^9$  spores/ml was added to an electroporation cuvette and exposed to square wave pulses.

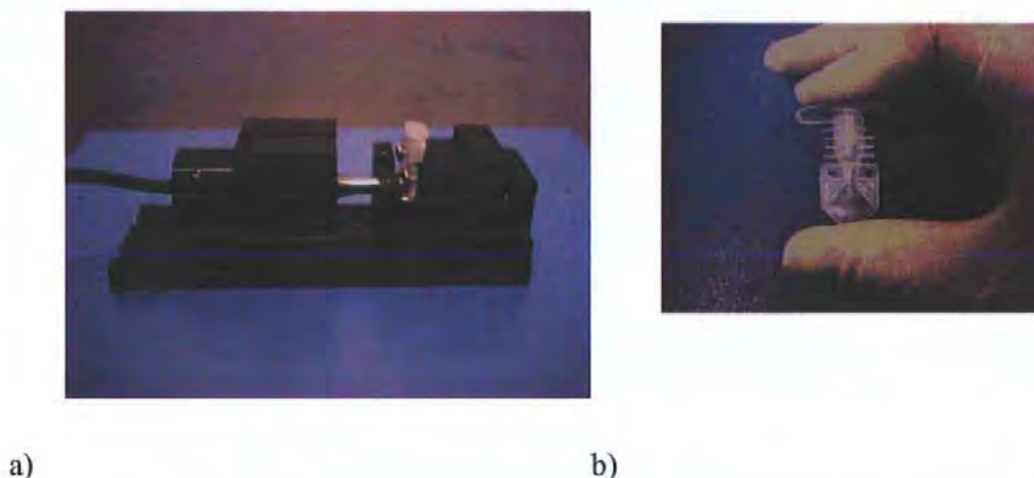
### **3.16 Sonication using a standard probe sonicator**

Initial evaluation of sonication was carried out using a standard probe sonicator, using a Misonix XL2020 (Misonix Inc) ultrasonic liquid processor. This system had a 550 watts output, with a 20 kHz converter and 1/2" tapped horn. A 3.2 mm microtip probe was used to maximise the amplitude of the sonication. The system was tuned according to the manufacturer's instructions.



### 3.17 Sonication using a mini sonicator (Cepheid)

A minisonicator system developed by Cepheid, (CA, USA) was supplied for assessment. The system utilised a 20 kHz minisonicator with a 6mm diameter titanium probe (Figure 3.2a). Batch samples of 100  $\mu$ l of spore suspension were aliquotted into Smartcycler tubes (Eurogentech), shown in Figure 3.2b (Cepheid, CA, US). The samples were exposed to cavitation, varying the amplitude and time period of the sonication. Samples were also sonicated with the use of acid washed glass beads (Sigma 0.05 mg, <106  $\mu$ m).



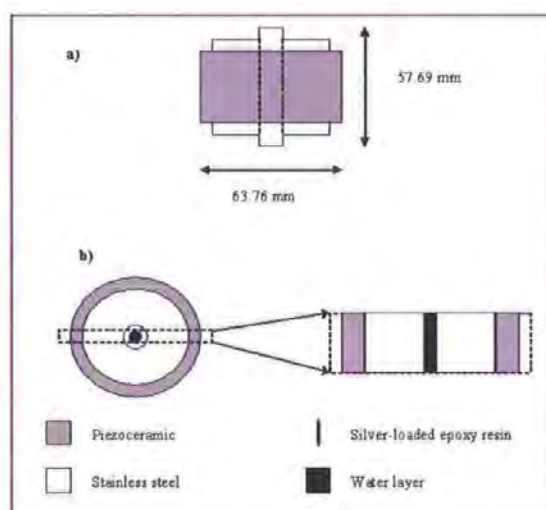
**Figure 3.1.** The Cepheid minisonicator showing: a) the sonicator with sample tube; b) the sample tube (holding 100  $\mu$ l of sample).

### 3.18 Development and evaluation of a prototype tubular sonicator

#### 3.18.1 Design

Using the preliminary results obtained using the standard probe and Cepheid system a continuous flow disruption system was developed in collaboration with K. Borthwick and T. Coakley, University of Cardiff. The design consisted of a system with a ring transducer and a stainless steel tubular coupling layer which allowed for heat dissipation, which was found to be a major factor in the degradation of the sensitivity of immunoassay based technique. A 26 mm long tubular ceramic transducer (PZT4D, Vernitron, Southampton, UK) of outer diameter 63.8 mm and wall thickness 6.52 mm (298 kHz radial resonance of the transducer) was fitted with a 50.5 mm outer diameter, 3.8 mm inner diameter steel

cylinder (Figure 3.2). The ceramic/steel bond was formed by silver-loaded conductive epoxy resin (Circuitworks ®). The epoxy-coated steel was held in a lathe and the transducer gently pushed on. A wave generator (Agilent model HP 33120A) provided an input voltage to an amplifier (Model 240L, ENI Rochester, NY) whose output was applied to the transducer.



**Figure 3.2. Schematic diagram of the tubular sonicator (a) vertical plane and (b) horizontal plane.** Image kindly provided by K. Borthwick, University of Cardiff.

### 3.18.2 Sample processing

Initial selection of the optimum frequency for cavitation and other preliminary measurements were conducted by K. Borthwick (University of Cardiff). The speed of sound, density, elastic stiffness constant and effective acoustic quality factors for the different layers were as detailed by Hawkes *et al.* (2002). A number of different voltage amplitudes were applied to the amplifier input at the selected frequency. This also involved determination of the optimal operating frequency for the system found to be 266 kHz, identified by a resonance peak. Further design of the system to introduce cooling through an increase in the steel heat radiating surface demonstrated a 5°C temperature rise over 60 s. Initial evaluation of disruption was assessed using *Saccharomyces cerevisiae* (conducted by K. Borthwick, University of Cardiff). After the preliminary evaluation, suspensions of

*B. atrophaeus* and *B. anthracis* UM23Cl2 spores were also subjected to cavitation in this system. The transducer was sealed at one end and 600 µl of washed *B. atrophaeus* spores at 10<sup>9</sup> cfu/ml were added, the top end sealed and the sample sonicated for increasing time periods. The transducer was washed extensively with sterile distilled water after sonication of each sample to avoid any carry over. The transducer was further developed into a flow through system. Flow rates were optimised for maximum sensitivity by Resonant Mirror immunoassay, as obtained for the batch system. Additional cooling was also integrated into the system using heat sinks and small cooling fans. Sample (5 ml) was introduced to the system using a pump and the sample sonicated at 267 kHz at a flow rate of 0.3 ml/min. Using this system a range of other parameters were tested, including the concentration of protein released, the effects of the application of ultrasound in relevant buffers and the effects of cavitation on the spore and proteins released.

### **3.19 Viable counts**

Serial tenfold dilutions of samples were prepared in sterile distilled water. The samples were plated in triplicate onto LB agar plates (100 µl of sample per plate). Plates were incubated overnight at 37°C and colony forming units (cfu) per ml calculated from the mean of the dilutions which gave a statistically significant number of colonies per plate (30 to 300).

### **3.20 Total counts**

A Fuchs Rosenthal haemocytometer was prepared, using a cover slip which was applied to produce characteristic Newton's rings. Spores were diluted in sterile distilled water and loaded into the counting chambers of the haemocytometer.

### **3.21 Electron microscopy**

All electron microscopy was conducted by J. Parkes or S. Smith, Dstl, with the exception of TEM images from immunogold labelling experiments which were undertaken personally.

### **3.21.1 Scanning Electron Microscopy (SEM)**

Briefly, 10 µl of sample was placed on a 0.1 µm nucleopore analysis membranes, allowed to air dry and coated with a conductive layer of gold. Images were obtained using a Hitachi S-800 Field Emission SEM operating at 5 kV.

### **3.21.2 Transmission Electron Microscopy (TEM)**

Samples were fixed for 18 h in 4% glutaraldehyde buffered in sodium cacodylate (pH 7.2), washed 3 times in buffer and post fixed in 1% buffered osmium tetroxide. Spores were dehydrated through a graded ethanol series and embedded in Spurr's resin. Ultrathin sections (50 nm) were stained with uranyl acetate and lead citrate and imaged using a Philips CM12 TEM. Photographs were recorded onto Kodak film and digitised at 800dpi using an Epson flatbed film scanner.

### **3.22 Determination of protein concentration**

Samples were centrifuged at 10000 g for 10 min and the supernatant was recentrifuged at 6000 g to remove any residual cells or spores. The protein concentration was determined using a standard or micro-BCA assay (Perbio) in a 96-well Immunolon 2HB microtitre plate (Nunc) according to the manufacturer's instructions.

### **3.23 Purification of proteins released**

Samples were centrifuged at 10000 g for 10 min and the supernatants removed and filtered through a 0.2 µm low-protein binding filter (Gelman Sciences). An aliquot was retained for analysis.

### **3.24 Concentration of proteins released**

Samples were concentrated using a 20 ml Omegacell with 5000 Da molecular weight cut-off (Sigma). Each sample was sterility checked to confirm the absence of viable spores.

The amount of protein in the samples was quantified using a BCA protein assay (Perbio) according to the manufacturer's instructions.

### **3.25 Immunoassays**

#### **3.25.1 Direct ELISA**

Aliquots (100 µl) of protein released from sonicated spores, EA1, S-layer extract (10 µg/ml in PBS) or *Bacillus* spores (1 x 10<sup>6</sup> spores/ml in water) and appropriate control antigens were coated onto Immulon2 HB 96-well microtitre plates (Nunc) and incubated overnight at 5 °C. The plates were washed 3 times with sterile phosphate buffered saline (PBS) containing 0.05% v/v polyoxyethylene sorbitan monolaurate (Tween 20) (PBST) followed by 1 x PBS (200 µl per well). Non-specific binding was blocked with 1% (w/v) milk powder in PBST, (100 µl per well) and left to incubate for 1 h at room temperature. The plates were washed 3 times PBST and 3 times PBS and an antigen specific antibody (polyclonal anti-*B. atrophaeus* or a monoclonal or anti-*B. anthracis* antibody, both prepared by Dstl) was added at a final concentration of 10 µg/ml in 1% (w/v) milk-PBST. A species-specific horseradish peroxidase conjugate (goat anti-rabbit HRP conjugate, or a goat anti-mouse-HRP conjugate, both Sigma) were added at a 1: 10000 or 1: 5000 dilution respectively in milk-PBST. Development buffer, composed of 50 ml ABTS buffer (0.1M Mn<sub>2</sub>HPO<sub>4</sub> Citric acid pH4.3, 1 ABTS tablet (2,2-Azino-bis-3-ethylbenzothiazoline-6sulphonic acid) and 15µl hydrogen peroxide) was added (100 µl per well) and left to incubate until sufficient colour had developed. Bound antibody was quantified by measuring the conversion of ABTS substrate to coloured product based on A<sub>414</sub> readings in an automated ELISA reader (Anthos 2001, Anthos Labtec Instruments, Salzburg, Austria). Negative controls were incorporate to ascertain background readings. In the case of ELISA using scFv, polyethyleneglycol (PEG) -purified phage-displayed scFv were diluted with MPBS, and an anti-M13 HRP conjugated antibody (Sigma) used to detect bound phage.

#### **3.25.2 Capture/sandwich ELISA**

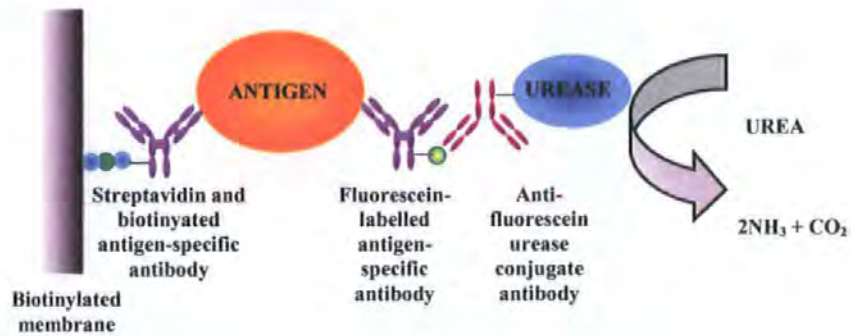
Microtitre plates (Nunc) were coated in aliquots (100 µl) of antigen specific antibody diluted in PBS at a final concentration of 10 µg/ml and incubated overnight at 5°C (per well). The plates were washed and blocked as described previously (section 3.25.1) and antigen added at a known concentration in 1% (w/v) milk-PBST (100 µl per well) and the

plates left to incubate for one hour at room temperature, with shaking. The plates were washed 3 times in PBST and once in PBS (200  $\mu$ l per well). A secondary antigen specific biotinylated antibody (that was prepared using a MCR of 10 biotin to 1 antibody (as described in section 3.14) was added at a final concentration of 10  $\mu$ g/ml in 1% (w/v) milk-PBST (100  $\mu$ l per well) and left to incubate for 1 h at room temperature, with shaking. A streptavidin HRP conjugate (Sigma) was then added at a final concentration of 10  $\mu$ g/ml in 1% (w/v) milk powder in PBST (100  $\mu$ l per well) and left to incubate for 1 h at room temperature, with shaking. Bound antibody was quantified by measuring the conversion of ABTS substrate to coloured product based on  $A_{414}$  readings in an automated ELISA reader (Anthos 2001). Negative controls were incorporated to ascertain background readings. A positive was defined as the background plus two standard deviations from the mean.

### **3.25.3 Threshold<sup>®</sup> assay**

The Threshold system was developed by Molecular Devices is an antibody based system which uses a light addressable potentiometer (LAP) to detect a localised pH change produced when antigen binds to antibody. The system works in a sandwich format and is shown diagrammatically in Figure 3.3.





**Figure 3.3 The Threshold<sup>®</sup> Assay.** A complex is formed between the streptavidin, biotinylated antibody, the antigen and the fluorescein antibody; the reagent mix. This is then allowed to bind to the biotinylated membrane. A second anti-urease FITC conjugate is added. The biotinylated membrane (assay stick) is then placed into the reader that contains urea. The urea is hydrolysed by urease to form ammonia and carbamate. The carbamate then immediately hydrolyses to form carbonic acid and two molecules of ammonia. At the physiological pH of the reader the ammonia equilibrates with water and carbonic acid proton dissociates finally resulting in two molecules of ammonia and one of carbon dioxide. This causes a net increase in pH within a small volume, which is measured by the sensor.

The Threshold<sup>®</sup> reader was rinsed with water and filled with 28 ml substrate solution (0.6 g per 100 ml urea) using a Luer-Lok syringe. Assay buffer (100  $\mu$ l) composed of 10 mM potassium phosphate, 154 mM sodium chloride, 0.005% (w/v) sodium azide, 0.025% (v/v) Triton-X 100, 0.1% (w/v) bovine serum albumin, pH 7.0, was added to all sample and positive control wells. Assay buffer (200  $\mu$ l) was added to all negative control wells and 100  $\mu$ l of sample to the appropriate sample wells. For each Threshold<sup>®</sup> assay stick (Molecular Devices) the reagent mix was prepared as follows: 30  $\mu$ l polyclonal goat anti-Ames antibody (prepared using a MCR of 10 biotin to 1 antibody, section 3.14) or 30  $\mu$ l polyclonal rabbit anti-*B. atrophaeus* antibody (prepared using a MCR of 5 biotin to 1 antibody, section 3.14) at 100  $\mu$ g/ml in assay buffer, 30  $\mu$ l polyclonal goat anti-Ames antibody (prepared using an MCR of 5 fluorescein to 1 antibody, section 3.14) or polyclonal rabbit anti-*B. atrophaeus* (prepared using an MCR of 30 fluorescein to 1 antibody, section 3.14) at 100  $\mu$ g/ml in assay buffer, 100  $\mu$ l of streptavidin, at 1 mg/ml and 840  $\mu$ l of assay buffer. An aliquot of reagent mix was added to all wells, 100  $\mu$ l of reagent mix was added to all sample wells and 200  $\mu$ l of reagent mix was added to all negative

control wells. The samples in all wells were mixed and 100  $\mu$ l was transferred to a Threshold<sup>®</sup> assay stick that was placed on the instrument filter block, and left to incubate for 1 min. The vacuum was set to low and the reagent allowed to filter through. An anti-FITC urease conjugate (100  $\mu$ l, from a stock prepared by reconstitution of the anti-FITC urease conjugate with 30 ml assay buffer per vial Molecular Devices) was added to each well and again allowed to filter through. Finally 400  $\mu$ l of wash buffer (10 mM potassium phosphate, 100 mM sodium chloride, 0.05% (v/v) Tween 20, 0.005% (w/v) sodium azide, 0.5 mM tetrasodium EDTA, pH 6.5) was added to each well and the vacuum set to high and allowed to filter through. The assay stick was released from the filter block and placed in a beaker of wash buffer and then read. Each assay was performed in triplicate and negative controls were incorporated to ascertain background readings. A positive was defined as the background plus two standard deviations from the mean.

#### **3.25.4 Lateral flow assays**

Lateral flow assays are antibody based and can detect antigen following application of the sample into a small well at one end of a membrane strip. The sample then wicks down the membrane strip and positive identification is shown as a visible line on the membrane strip, produced from the reaction of a specific antigen conjugate (often chromogenic) with the antigen. A second line of antibody directed against the conjugate acts as a positive control. Samples were diluted as appropriate and three drops of sample placed in the sample well of the lateral flow assay, using a disposable Pasteur pipette. The assay was incubated at room temperature for a maximum of 20 min; each assay was performed in triplicate.

#### **3.25.5 Specific near real time detection using the resonant mirror biosensor**

##### ***3.25.5.1 Antibody immobilisation***

Antibodies were immobilised onto carboxymethylated dextran cuvettes (Affinity Sensors) using standard amine coupling methods. Briefly, the surface was washed 3 times with 170  $\mu$ l PBST and was activated using 3 x 170  $\mu$ l 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 0.05 M N-hydroxysuccinimide (NHS) for a defined time period. The activation time was 8 min for the high (T500) and 5 min for the low molecular weight (T70) carboxymethylated dextran cuvettes. These times



were found to be optimal for ligand loading and subsequent detection of antigen in preliminary experiments. Following activation, the surface was washed 3 times with 170  $\mu$ l PBST, then 3 times with 170  $\mu$ l 10 mM acetate buffer, pH 4.5. The antibody was then added to the acetate buffer at a final concentration of 100  $\mu$ g/ml for 15 min and the surface was washed with 3 x 170  $\mu$ l PBST. Finally, unreacted groups were blocked with 1 M ethanolamine pH 8.5 and the surface washed 3 x 170  $\mu$ l PBST.

#### **3.25.5.2 Binding protocol**

The sensor surface was washed with 3 x 170  $\mu$ l PBST and the baseline allowed to establish. Antigen (17  $\mu$ l) was added to the surface and left for 10 min. The surface was washed 3 x 170  $\mu$ l PBST. The surface was regenerated with 20 mM KOH for 3 min (*B. atrophaeus* spores) or 10 mM KOH for *B. anthracis* spores. The surface was washed 3 x 170  $\mu$ l PBST. The protocol was repeated twice so each concentration was performed in triplicate. Negative controls were included to assess any non-specific binding including a non-specific antibody and a surface that had been activated and blocked.

#### **3.26 Evaluation of 2,6-pyridinedicarboxylic acid (DPA) release**

The DPA assay was based on that reported by Hindle and Hall (1999), optimised for the detection of spores and reporting a limit of detection of 2 nM DPA. Stock solutions of 1 mM DPA (Sigma), 1 mM TbCl<sub>3</sub> (Aldrich) and 0.1 M acetate buffer pH 5.6 (Sigma) were prepared. Dilutions of DPA were prepared from 1 nM - 25  $\mu$ M in order to obtain a standard curve. All measurements were taken using a Perkin Elmer LS55 Luminescence spectrophotometer, using WinLabFL software. The assay was optimised for the instrument and the final parameters used were an excitation wavelength of 275 nm, a delay of 0.1 ms, a gate of 9.9 ms and the detection photo multiplier tube (PMT) set to 775 V. Assay reagents were then made up consisting of 3.6 ml 0.1 M acetate buffer and 400  $\mu$ l of 1 mM TbCl<sub>3</sub>, 2.95 ml was then transferred to a quartz fluorescence cuvette and 50  $\mu$ l of DPA standard or sample was added. A total of 10 readings taken for each individual assay, each of which was preformed in triplicate.

### 3.27 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

The range of proteins released during sonication, was evaluated based on the observed differences in molecular weight by SDS-PAGE using Tris-glycine or NuPAGE® Novex Bis-Tris precast gels (Invitrogen), according to the manufacturer's instructions. Briefly for Tris-glycine gels samples were prepared by adding 15 µl of 2 x Laemmli sample buffer (Sigma) to 15 µl of sample. Sigma wide range molecular weight marker (5 µl) was added to 5 µl 2 x Laemmli sample buffer; ovalbumin (5 µg) was added as a control. The samples were incubated in a boiling water bath for 4 min and then held briefly on ice, and pulsed in a microfuge at 13000 g. Samples (20 µl) were loaded into the appropriate wells and the gels run according to the manufacturer's instructions. In the case of Bis-Tris Gels, for each 20 µl sample the following reagents were added: 5 µl 4x LDS Sample buffer (Invitrogen), 2 µl 10x reducing agent (Invitrogen) and 13 µl of sample. Samples were incubated at 70°C for 10 min and pulsed in a microfuge at 13000 g. Samples (20 µl) were then loaded into the appropriate wells and the gels run at the appropriate voltage and time period according to the manufacturer's instructions. The gels were removed from the cassettes and fixed and stained appropriately using Colloidal Blue or SilverQuest™ Silver staining kit (Invitrogen) according to the manufacturer's instructions or used for western Blot analysis. In the case of immunopurified proteins from Dynal anti-species beads the bound proteins were eluted into 1x LDS reducing sample buffer (Invitrogen) by incubation in a water bath at 70°C for 10 min.

### 3.28 Western blot analysis

Identification of the proteins recognised by antibody was carried out by western blot analysis. Samples separated by SDS-PAGE were transferred onto polyvinylidene difluoride (PVDF) or 0.2 µm nitrocellulose membranes (both Invitrogen) according to the manufacturer's instructions. After blocking the membranes overnight in PBST containing 5% (W/V) milk powder PBS 0.1% Tween 20 (v/v) blots were rinsed briefly PBS 0.1% v/v Tween 20 (PBST) and incubated in 5% milk powder PBST, The membranes were then probed using an anti-*B. atrophaeus* antibody (Dstl) at 5 µg/ml, or scFv antibody at 2.5 µg/ml in 3% (w/v) MPBST for 1 h at room temperature with shaking. Blots were washed as described previously and incubated in an anti-His HRP conjugate (Sigma) at 1:1000

dilution and an anti-rabbit HRP conjugate (Amersham) antibody at 1:1000 dilution in 3% (w/v) MPBST for 1 h at room temperature with shaking. In the identification of immunogenic proteins of the exosporium the primary antibody was a biotinylated polyclonal rabbit anti-Ames antibody at a concentration of 5 µg/ml and the secondary antibody streptavidin HRP conjugate (Sigma) at 1:1000 or an anti-rabbit HRP conjugate (Amersham BioSciences) antibody at 1:10000 dilution in 3% (w/v) milk powder PBST for 1 h at RT with shaking. To identify biotinylated surface located proteins only a streptavidin HRP conjugate (Sigma) at 1:1000 in 3% (w/v) milk powder PBST was used. For monoclonal antibody evaluation, all anti-EA1 mAbs used as the primary antibody were used at a final concentration of 5 µg/ml and an anti-mouse HRP conjugate (Amersham) was used at 1:5000 dilution both in 3% milk powder PBST. For determination of molecular mass, MagicMark™ Western protein standard (Invitrogen) was used. Blots were washed 1 x 15 min and 4 x 15 min in PBST. Protein bands recognised by the antibody were visualised by enhanced chemiluminescence (ECL Detection reagent kit, Amersham) and exposed to ECL hyperfilm (Amersham) according to the manufacturer's instructions.

### **3.29 Two-dimensional (2D) electrophoresis**

Sample rehydration buffer (8M urea, 2% CHAPS, 0.5% (v/v) carrier ampholytes (pH 3-10), 0.002% Bromophenol Blue), containing protein sample was loaded into a sample loading well of a Zoom IPG cassette (155 µl). Initially a wide pH range was chosen, in order to determine which narrower pH range would be more appropriate. A pH 3-10 zoom strip was placed into the zoom cassette (acidic end first) and the loading wells sealed. The samples were incubated for 16 h at room temperature. The sealing tape and loading wells were removed, a wick placed at each end of the cassette and 750 µl of deionised water was applied to each electrode and the excess blotted off. The IPG cassette was placed into the ZOOM® IPG Runner™ Buffer Core and the outer chamber filled with 600 ml of deionised water. The samples were run at 500 V for 3-4 h, using a PowerEase®500 Power supply. The IEF strips were removed from the cassette for subsequent electrophoresis. LDS sample buffer 4x (Invitrogen) was diluted to 1x with deionised water (10 ml per strip). 500 µl of NuPAGE® Sample Reducing Agent (10x) was added to 4.5 ml of 1x NuPAGE® agent and placed in a 15 ml Falcon tube and an IPG strip added and incubated for 15 min at room temperature. The IPG strip was removed from the Falcon tube and placed into an 4-20% IPG ZOOM® gel well and 400 µl of 0.5% agarose added and allowed to solidify. Wide Range molecular weight marker (Sigma) was loaded onto the gel and the gel was run at

200 V for 50 min using 1 x MES running buffer. The gel was removed from the cassette and fixed and stained using a SilverQuest stain (Invitrogen) according to the manufacturer's instructions.

### **3.30 Identification of the immunogenic proteins of *B. anthracis* exosporium**

The antibody used for all attachment strategies was a polyclonal rabbit anti-exosporium antibody (Dstl).

#### **3.30.1 Column purification of immunogenic *B. anthracis* exosporium proteins**

Antibodies were coupled to the matrix of a 1 ml HiTrap™ N-hydroxysuccinimide (NHS)-activated column HP column (Amersham Biosciences) according to the manufacturer's instructions. The antibody was dialysed into coupling buffer (0.2 M sodium bicarbonate, 0.5 M sodium chloride pH 8.3). The column was washed with 6 ml ice-cold HCl and then the antibody was loaded onto the column at 5 mg/ml in coupling buffer and allowed to incubate for 1 h at room temperature. The column was washed with 6 ml of buffer A (0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 and then 6 ml of buffer B (0.1 M acetate, 0.05 M NaCl, pH 4.0) and then a further 6 ml of buffer A. After a further incubation at room temperature for 30 min the column was washed with 6 ml buffer B, 6 ml buffer A and finally 6 ml buffer B. Purification of spore proteins was then undertaken. The column was washed with 5 ml PBS and then 1 ml of sample was loaded and the column allowed to incubate at room temperature for increasing time periods. The column was washed with 10 ml PBS and then eluted with 10 mM KOH (shown to be suitable for regeneration using the evanescent wave biosensors). To neutralise the elutions 16 µl of 1 M HCl. The elution of proteins was monitored using the absorbance at 280 nm and samples were also analysed by SDS-PAGE.

#### **3.30.2 Bead-based purification of immunogenic *B. anthracis* exosporium proteins**

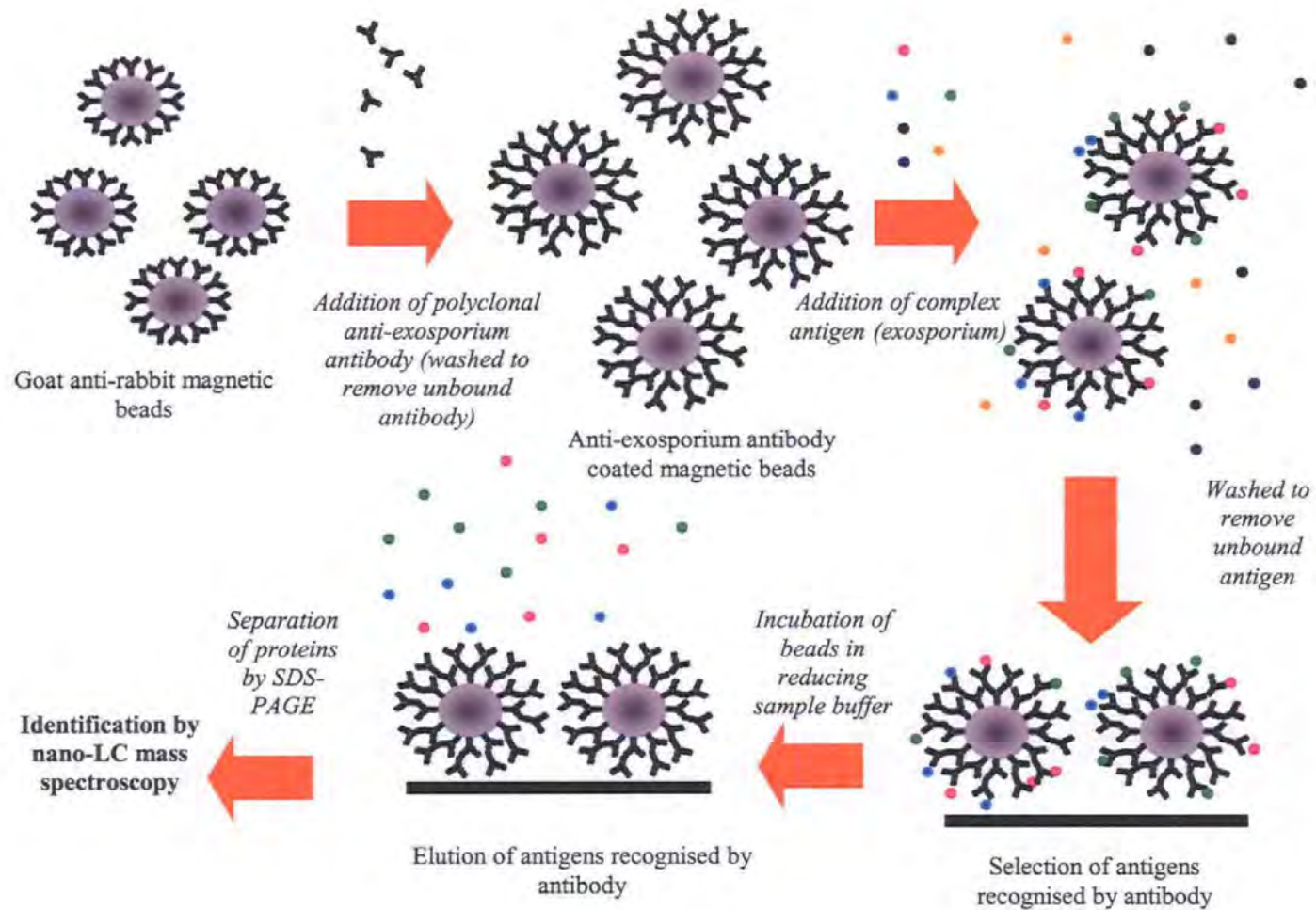
##### **3.30.2.1 Non-directed covalent coupling**

This was carried out with the use of a goat anti-*B. anthracis* (Ames) antibody to capture those proteins that were immunogenic on Magnabind™ carboxylated derivatised beads (Perbio). The antibody was immobilised onto the beads by washing the beads 3 times in PBS then adding 500 µl of 0.2 M EDC/ 0.05 M NHS per 200 µl of beads. The beads were

incubated for 30 min at room temperature with mixing and then washed 3 times with PBS. Antibody was added (500  $\mu$ l) at a final concentration of 1 mg/ml in sterile PBS and the beads incubated overnight at 2-8°C. The efficiency of antibody binding was calculated by measuring the absorbance at 280 nm of the antibody solution before immobilisation and of the wash fractions after binding. The beads were then washed three times with PBS and 500  $\mu$ l of 1 M ethanolamine pH 8.5 added and incubated for 10 min at room temperature. The beads were washed 5 times in PBS. An aliquot (500  $\mu$ l) of sonicated spore supernatant (from spores sonicated at  $\sim 1 \times 10^9$  cfu/ml) was added and incubated for three hours at room temperature. Any bound proteins were eluted using 500  $\mu$ l of 10 mM KOH for five min. To neutralise the elutions 8  $\mu$ l of 1 M HCl was added. The sample buffer was exchanged for PBS and the samples concentrated using Vivaspin500 columns. The samples were analysed by SDS-PAGE and subsequent silver staining (section 3.27).

#### ***3.30.2.2 Site-directed binding***

Immunopurification using DYNAL anti-species beads was found to yield the most successful results for protein purification. The immunopurification method devised for the identification of immunogenic proteins is represented diagrammatically in Figure 3.3.



**Figure 3.4 Method developed for the identification of immunogenic proteins .**

Magnetic anti-species beads were obtained from DYNAL (sheep anti-rabbit IgG Dynabeads™ M-280). To wash the beads, 250 µl of bead stock ( $1.675 \times 10^8$  beads) was aliquoted into a tube and placed into a magnetic particle concentrator (MPC) (Dyna) for 2 min. The supernatant was removed and the beads resuspended in 250 µl PBS; 0.1% (v/v) Tween 20. This washing step was repeated twice. Rabbit anti-exosporium antibody was added to a 250 µl aliquot of beads and left to incubate overnight with rotation at room temperature. To remove unbound antibody the beads were washed three times with 1 ml volumes of PBS; 0.1% (w/v) BSA, using the MPC to separate the beads from the liquid as described previously. In order to cross-link the immobilised antibody, the beads were resuspended in 10 ml of 20 mM dimethyl pimelimidate (Perbio) in 0.2 M ethanolamine pH 9.5 (Sigma) and incubated for 45 min at room temperature with rotation. The tubes were placed into the MPC, the supernatant pipetted off and the beads resuspended in 10 ml of 0.2 M ethanolamine pH 9.5 for 3 h with rotational mixing at room temperature. The beads were then washed 3 times using the MPC, once with 10 ml PBS, once with 10 ml PBS, 0.01% (v/v) Triton X-100 to remove non-covalently bound antibody and then finally with 10 ml PBS. The beads were then incubated with the appropriate antigen or control, in sterile PBS, 0.1% (v/v) Tween 20, 0.1% (w/v) BSA. The following controls were incorporated: (a) beads with immobilised antibody with no antigen; (b) beads with no immobilised antibody incubated with antigen; and (c) beads with no immobilised antibody or antigen. Unwashed *B. anthracis* wild type Ames exosporium was used as the antigen at a concentration of approximately 10 µg/ml in sterile PBS, 0.1% (v/v) Tween 20, 0.1% (w/v) BSA. The beads were left to incubate overnight at room temperature with rotation. To remove unbound proteins, the beads were washed 3 times, twice with 1 ml volumes of sterile PBS pH 7.4; 0.1% (v/v) Tween 20 and once with 1 ml PBS only using the MPC. The proteins were then eluted directly by incubating the beads in 50 µl LDS reducing sample buffer (Invitrogen) for 10 min at 70°C. The samples were centrifuged briefly and separated from the beads by placing the tubes into the MPC for 2 min. The samples (supernatants) were subsequently analysed by SDS-PAGE on a 4-12% pre-cast Bis-Tris gel (Invitrogen) at 125 V for 40 min according to the manufacturers instructions. After visualisation with Colloidal Blue Stain (Invitrogen), each protein band of interest was excised and subjected to *in situ* trypsin digestion using the method of Shevchenko *et al.* (1996). The resulting peptides were separated by nano-reversed phase liquid chromatography using a Waters C18, 3 µm, 100Å (150 mm x 75 µm, i.d.) column, and electrosprayed into a quadrupole time-of-flight tandem mass spectrometer (section 3.31).

### **3.31 Mass spectrometry (MS) and data processing of immunogenic exosporium proteins**

MS was kindly conducted by P. Skipp (University of Southampton). All data were acquired using a Q-tof Global Ultima (Waters Ltd) fitted with a nanoLockSpray™ source to achieve better than 10 ppm mass accuracy. A survey scan was acquired from  $m/z$  375 to 1800 with the switching criteria for MS to MS/MS including ion intensity and charge state. The collision energy used to perform MS/MS was varied according to the mass and charge state of the eluting peptide.

All MS/MS spectra were automatically processed using MassLynx 4.0 (Waters Ltd) and searched against the NCBI non-redundant database (June, 2004 versions), using ProteinLynx Global Server 2.05. Proteins were only assigned if, for each peptide ion, greater or equal to three experimentally derived  $y$  ions could be matched to the predicted spectra.

### **3.32 Identification of other *B. anthracis* spore proteins**

#### **3.32.1 Digestion of the supernatant from sonicated spores**

Washed or Urografin purified spores were sonicated and the proteins separated, concentrated and quantified as described previously. The protein sample (45  $\mu$ l) was combined with 2.5  $\mu$ l of a 0.25 mg/ml solution of pepsin (Sigma) in water to give a substrate to enzyme ratio of 24:1. Formic acid was then added to give a final concentration of 5% (v/v). Solutions were incubated at 37°C for increasing time periods. Resulting mixtures were stored at -20°C until required.

#### **3.32.2 In-gel digestion of proteins from sonicated spores.**

Proteins released from spores by sonication were separated on 4-12% BIS-TRIS gels (Invitrogen) and stained using a Colloidal Blue Stain (Invitrogen). Selected bands were excised from the gel and transferred to individual microfuge tubes containing 5% (v/v) acetic acid. In gel digestion and subsequent MS analysis was kindly conducted and P. Skipp (University of Southampton), as described previously (3.31), or by K. Anderson (Dstl). Briefly, the acetic acid was removed from the samples and washed for 2 x 5 min in



100  $\mu$ l sterile distilled water. The liquid was removed and the samples washed for 2 x 15 min in 100  $\mu$ l water:acetonitrile 1:1 (v/v). The liquid was removed and washed for 5 min in acetonitrile. The liquid was removed and the sample rehydrated with 100  $\mu$ l 0.1 M ammonium bicarbonate for 5 min and then an equal volume of acetonitrile added and incubated for 15 min. The liquid was removed and the sample dehydrated for 5 min in 100  $\mu$ l acetonitrile. After removal of all stain all liquid was removed and the samples vacuum dried for 30 min (Concentrator 5301, Eppendorf). The gel sections were then rehydrated in 100 $\mu$ l dithiothreitol in 0.1 M ammonium bicarbonate and incubated for 45 min at 56°C. The samples were then cooled to room temperature and the liquid removed, 100  $\mu$ l of 55mM iodoacetamide in 0.1 M ammonium bicarbonate was then added and incubated in the dark for 30 min. The liquid was removed and the samples washed for 5 min in 0.1 M ammonium bicarbonate. An equal volume of acetonitrile was added and incubated for 15 min and then the liquid removed and the sample dehydrated in 100  $\mu$ l acetonitrile for 5 min. All the liquid was removed from the samples and then they were vacuum dried for 30 min. The samples were rehydrated in 50  $\mu$ l digestion buffer with trypsin (0.1 mM calcium chloride, 50 mM ammonium bicarbonate, 12.5 ng/ $\mu$ l trypsin (sequencing grade, Promega) and incubated on ice for 45 min. Digestion buffer (0.1 mM calcium chloride, 50 mM ammonium bicarbonate) was added (75  $\mu$ l) and incubate overnight at 37°C. Peptides were extracted by adding 60  $\mu$ l 25 mM ammonium bicarbonate and incubated for 15 min. An equal volume of acetonitrile was added and incubated for a further 15 min. The liquid containing the peptides was then transferred to a new tube and 100  $\mu$ l of 5% (v/v) formic acid: acetonitrile, 1:1 (v/v) added to the remaining gel samples and incubated for 15 min the liquid was then added to the first extracted peptides and the extraction of the peptides in the gel sections repeated and added to the previous fractions. The pooled fractions were vacuum dried and stored at -20°C until required for further analysis. Prior to MS analysis the digest mixtures were purified using a c18 ZipTip (Millipore), according to the manufacturer's instructions. The sample was added to 2  $\mu$ l 2.5% (v/v) TFA before binding to the ZipTip to give a final volume of 4  $\mu$ l.

### **3.32.3 LC-MS/MS of enzymatically digested sonicated spore supernatants**

Spore proteins from washed and Urografin purified spores were prepared and digested as described previously. LC-MS/MS analysis was kindly conducted by K. Anderson (Dstl) using an LQA DECA ion trap mass spectrometer. Briefly, LC separation utilised a Jupiter

(Phenomemex, Macclesfield, UK) C18 column (150 x 1 mm, 5  $\mu\text{m}$ , 300Å). Solvent delivery was provided by an HP1100 Series LC system (Agilent Technologies UK Ltd., Stockport, UK). A linear gradient elution was conducted at a flow rate of 50  $\mu\text{l}/\text{min}$  with a mobile phase comprising solvent A (0.05% (v/v) TFA in water) and solvent B (0.05% (v/v) TFA in acetonitrile). The gradient used was a hold at 5% B between 0 and 10 min, a linear increase to 15% B at 80 min, a linear increase to 65% B at 90 min and finally a linear increase to 90% B at 95 min. Subsequently 90% B was held until 97 min and a return to 5% B at 100 min. The column was then re-equilibrated at 5% B to 145 min. Sample volumes used were between 20 and 50  $\mu\text{l}$ . The ion trap spectrometer was fitted with electrospray ionisation interfaces operated in positive ion mode. A spray voltage of 5 kV was applied using a capillary temperature of 275°C, sheath gas flow at 20 (arbitrary units) and the auxiliary gas set to 0 (arbitrary units). Three scan events were conducted, one full scan of mass spectra from  $m/z$  400 to 2000, scan two was a data dependent zoom scan (scan width  $\pm 5.0 m/z$ ) and the third scan event collected data-dependent MS/MS ion spectra. The raw data were converted into DTA file format and individual files from an LC separation merged to a single file for submission to the Mascot database.

#### 3.32.4 Nanospray analysis of in-gel digests

These were kindly carried out by K. Anderson (Dstl) using the Quattro II tandem quadrupole mass spectrometer fitted with a Z-spray ionisation source. Briefly, 2  $\mu\text{l}$  of sample was loaded onto a borosilicate glass capillary. Spray was initiated by application of gentle back pressure from an air filled syringe using a capillary voltage of 1.25 kV and a source block temperature of 80°C. Cone voltage was ramped from 35kV at  $m/z$  300 to 65 V at  $m/z$  1300. Spectra were scanned from  $m/z$  300 to 1300 over 10 s. Approximately 30 scans were averaged to give the final spectrum for each sample. Selected ions were subject to MS/MS analysis using the average of approximately 30 to 50 scans to produce the product ion spectrum for each precursor ion. Two databases were used (Mascot and Profound) to search the National Centre of Biotechnology Information (NCBI) sequence database (<http://www.ncbi.nih.gov/>). Entered search parameters were determined by the mass spectrometer utilised for the analysis (e.g. mass tolerance values) and known sample treatment.

### 3.33 Construction and use of immune mouse scFv library

The scFv library was kindly produced and provided by Dr Carl Mayers (Dstl). Briefly six 12 week old female Balb/c mice were immunised with  $\gamma$ -irradiated *B. anthracis* Ames spores. Each immunisation consisted of  $1 \times 10^7$  spores in Freund's incomplete adjuvant. Mice were immunised 4 times at intervals of three weeks, and killed by cervical dislocation once they showed a sufficiently high titre ( $>1:100\ 000$ ) to the spores by endpoint ELISA. Spleens were removed from the killed mice and splenic mRNA isolated using Trizol reagent (Invitrogen). The total RNA from the immunised mice was used to produce the immune scFv library; PCR amplification of antibody sequences, overlap extension PCR, cloning of the assembled scFv sequence into pAK100 and production of phage-displayed scFv was carried out by Mayers as described by Krebber *et al.* (1997).

### 3.34 Biopanning with EA1

Immunotubes (Nunc, UK) were coated with 1 ml of purified EA1 at 10  $\mu\text{g/ml}$  in phosphate buffered saline (PBS) overnight at 4  $^{\circ}\text{C}$  and blocked with 2 % (w/v) milk powder PBS (MPBS). 100  $\mu\text{l}$  of scFv-phage were mixed with 900  $\mu\text{l}$  MPBS, incubated for 1 h at room temperature, and added to the coated immunotubes. After a further 2 h room temperature incubation, the immunotubes were washed 10 times with PBS 0.1 % (v/v) Tween 20. Bound phage were eluted with 100 mM triethylamine and neutralised with 500  $\mu\text{l}$  1 M Tris HCl pH 7.5. Eluted phage were infected into log phase XL1-Blue *E. coli*, and plated on a 24 cm square 2 x YTGC (6 g/l peptone from casein; 10 g/l yeast extract, 5 g/l NaCl, 1% (w/v) glucose, 30  $\mu\text{g/ml}$  chloramphenicol) agar plate and incubated overnight at 30  $^{\circ}\text{C}$ . This procedure was repeated for each round of panning carried out. Competitive panning was carried out in an identical fashion, adding S-layer extracts to the scFv-phage MPBS solution for 1 h before panning. The concentrations of antigen used for competitive panning were 50  $\mu\text{g/ml}$  *B. cereus* 11145 S-layer protein, and 25  $\mu\text{g/ml}$  of *B. cereus* 11143, *B. cereus* 9946 and *B. pumilus* S-layer protein. The concentration of EA1 used for panning was 1 or 10  $\mu\text{g/ml}$ .

### **3.35 DNA fingerprinting of scFv clones**

scFv sequences from selected clones were amplified using PCR primers surrounding the scFv sequence (scfor and scback; Krebber *et al.*, 1997). Briefly, each 50  $\mu$ l PCR reaction comprised; 0.5  $\mu$ l scfor primer, 0.5  $\mu$ l scback primer, 5  $\mu$ l 10x PCR buffer (Invitrogen), 1  $\mu$ l 10mM dNTPs, 1.5  $\mu$ l 50 mM MgCl<sub>2</sub>, 0.5  $\mu$ l Platinum Taq (Invitrogen) and 41  $\mu$ l sterile H<sub>2</sub>O. The PCR was run for 32 cycles of 94°C for 60 sec, 62°C for 30 sec and 72°C for 60 s. All products were resolved on 2% E-gels (Invitrogen) using 100 bp markers according to the manufacturer's instructions. Those clones containing an 800 bp insert were positive for the scFv DNA insert and subjected to BstN1 digestion. A 100  $\mu$ l reaction comprised 10  $\mu$ l 10x NEB #2 reaction buffer (New England BioLabs), 10 mg/ml BSA, 35  $\mu$ l PCR product, 51  $\mu$ l sterile H<sub>2</sub>O; this was then incubated at 60°C for 3 h. Restriction digest products were resolved on 4% E-gels (Invitrogen) using 25 bp markers. The PCR and DNA fingerprinting were carried out on 10 randomly selected scFv from rounds 1 and 2 and 50 scFv clones from round three.

### **3.36 Obtaining the protein sequences for Round 3 scFv**

Plasmid preparations were completed using the Mini Plasmid Spin Prep Kit (Qiagen), according to the manufacturers instructions. The scFv DNA was sequenced to confirm that they were unique. These sequences were used for further analysis. The scFv were sequenced using forward and reverse primers for the plasmid (Oswell, UK or MWG, Sweden). Sequencing data from the DNA of the scFv was translated to a protein sequence using the Expasy translation tool (<http://ca.expasy.org/tools/dna.html>). The correct reading frame was initially identified through the presence of GGGGS and consecutive repeats of this sequence required for the linker sequence between the V<sub>H</sub> and V<sub>L</sub> regions that make up the scFv. Heavy and light chain CDRs were identified using the Kabat definition, as described by Martin (2001). The heavy and light chain CDR sequences were compared for the scFv sequences and similarities identified.

### **3.37 Soluble expression of selected scFvs**

For soluble expression, the scFv backbones from round 3 phage were cut from the pAK100 vector used for biopanning and ligated into pAK 300 and pAK400 vectors.

Restriction digestion of the original scFv from the pAk100 plasmid was undertaken using Sfi I (NEB). Each 50  $\mu$ l reaction consisted of 2.5 $\mu$ l 10x NEB #2 reaction buffer, 0.5  $\mu$ l 100x BSA, 1  $\mu$ l Sfi I, 1  $\mu$ g of DNA to be cut, and sterile distilled H<sub>2</sub>O to 50  $\mu$ l. The restriction digests were incubated overnight at 37°C. Digestion of the pAK300 and 400 vectors, required for ligation of the scFv plasmid, was completed using 20  $\mu$ g of vector DNA in 200  $\mu$ l reaction volumes, all reagent volumes were scaled up accordingly.

### **3.38 Crystal violet purification of scFv and plasmid DNA**

Loading buffer (5  $\mu$ l) was added to each 25  $\mu$ l of sample and 30  $\mu$ l was loaded into each lane of a 1% Crystal violet gel. The gels were run at 80 V until adequate separation had occurred. The cut DNA was observed as clear bands against a purple background and the bands excised. The bands were weighed and purified using a SNAP™ gel purification Kit (Invitrogen) according to the manufacturer's instructions.

### **3.39 Ligation of scFvs into pAK300 and pAK400**

The cut inserts from the six isolated DNA sequences from the scFv produced by the competitive panning procedure were ligated into digested pAK 300 and pAK 400 vectors. Ligation reactions were set up consisting of 200 ng of purified Sfi I cut pAK 300 and pAK 400, 20 ng purified scFv fragment, 1  $\mu$ l (1unit) of T4 DNA ligase and 4  $\mu$ l 5 x Invitrogen DNA ligase buffer. Sterile distilled water was added to a final volume of 25  $\mu$ l. The ligation reactions were mixed well and incubated overnight at 16°C.

### **3.40 Electroporation of competent cells**

Electrocompetent *E. coli* XL1 Blue were prepared by adding 400  $\mu$ l of sterile dH<sub>2</sub>O to the plasmid containing the scFv insert and centrifuged in a microcon 30 (Sigma) spin column at 11000 g for 8 min at room temperature. This was repeated for a total of 3 washes. The

column was inverted and centrifuged at 960 g for 3 min to collect the desalted plasmid. An aliquot of plasmid (50  $\mu$ l) was added to the electrocompetent cells, placed into a prechilled electroporation cuvette and pulsed at 2500 V, 200  $\Omega$ , 25  $\mu$ F, 2 mm gap using the BioRAD electroporation system, noting the time constant (4.5-5 m/s). SOC medium (20 g/l Bacto-tryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 2.5 ml of 1 M KCl, 20% (w/v) glucose) (900  $\mu$ l) was added and incubated at 37°C for one hour with shaking. A ten fold serial dilution of transformed cells was plated onto 2xYT 1% glucose, 30  $\mu$ g/ml chloramphenicol and incubated overnight at 37°C.

### **3.41 Confirming the presence of the scFv insert in selected clones**

#### **3.41.1 Minipreps of transformed cells**

A single colony (5 for each insert and pAK300 and 400 vectors as a control) from the transformation was inoculated into 50 ml of LB with 1% glucose, 30  $\mu$ g/ml chloramphenicol and incubated overnight at 37°C. Glycerol stocks were also made (50% glycerol 2 xYTGC) and the remaining culture used for colony PCR as detailed previously. DNA fingerprinting was undertaken using BstNI as described previously.

#### **3.41.2 Expression and purification of hexa-histidine tagged scFvs**

A single colony was inoculated into 200 ml 2xYTGC for pAK 300 and pAK 400 vectors containing the scFv inserts and incubated overnight at 37°C with shaking (200 rpm). A 95 ml volume of 2xYTGC was inoculated with 5 ml of overnight culture incubated until an OD<sub>600</sub> of ~0.8 was obtained. The culture was centrifuged at 2500 g for 10 min at 5°C, and the pellet resuspended in 50 ml of expression media. Isopropyl-beta-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and incubated at room temperature overnight with shaking. The culture (1 ml) was centrifuged and the pellet stored at -20°C, for further analysis. The remaining culture was centrifuged at 3500 g for 10 min at 5°C and the pellet resuspended in 5% of the original volume of periplasmic extraction buffer (50 mM TrisHCl, 20% w/v sucrose, 1 mM EDTA). This was then incubated on ice with occasional shaking. The suspension was centrifuged at 14000 rpm for 30 min at 5°C. The supernatant contained the soluble periplasmic extract and the pellet contained the insoluble periplasmic material. Both fractions were stored for further analysis at -20°C.

### **3.41.3 Initial confirmation of the presence of soluble scFv in the soluble periplasmic fraction by ELISA**

Purified EA1 was coated onto ELISA plates at a final concentration of 10 µg/ml in PBS and incubated overnight at 2-8°C. The ELISA procedure described previously was followed (2.25.1) but a mouse anti-His HRP conjugate was used as the secondary antibody (1:1000).

### **3.42 Purification of hexa-histidine tagged scFv**

The purification of the scFv was achieved using B-PER 6xHis Spin Purification kit (Perbio). An aliquot (1 ml) of 50% nickel chelated agarose slurry was added to each 10 ml of periplasmic extract and incubated at room temperature on a rotary mixer for one hour. The suspension was centrifuged at 1200 g for 10 min. The supernatant was retained for analysis and the pellet resuspended in 500 µl of buffer per 1 ml of agarose slurry that was initially added. The suspension was transferred to a spin column and centrifuged at 10000 g for 10 min at room temperature. Wash buffer (500 µl) was added and incubated for 5 min at room temperature and then centrifuged for 2 min at 10000 g. This was repeated. Finally, the collection tube was changed and the protein eluted by the addition of 0.5 ml of elution buffer, incubation for 5 min at room temperature and centrifugation for 2 min at 10000 g. The elution was repeated three times for a total of four elutions. All samples were kept for further analysis at -20°C.

### **3.43 Analysis of purified samples**

Samples were analysed on 4-12% Bis-Tris NuPage gels (Invitrogen) (section 3.27) and blotted onto nitrocellulose membranes (Invitrogen) according to the manufacturer's instructions (section 3.28). Gels were stained using a colloidal blue stain (section 3.27) and HIS tagged proteins were identified by western blot analysis (section 3.28) with the use of a mouse anti-His HRP conjugate (1:2000) using the ECL™ detection system (Amersham).

Those fractions containing the purified scFv were pooled and the buffer was exchanged for sterile PBS using a Vivaspin500 column (Sigma) according to the manufacturer's

instructions. The concentration of scFv in the final sample was quantified using a Micro BCA Protein Assay (Pierce).

#### **3.44 Kinetic analysis of scFv binding**

The kinetic data for scFv binding purified EA1 was obtained using the BIAcore 3000 (BIAcore, Sweden) with EA1 immobilised onto a CM5 sensor chip, and *B. cereus* 11145 S-layer protein as a negative control. Approximately 1500 Response Units (RU) was immobilised onto the surface using standard amine coupling and unreacted sites blocked with 1M ethanolamine, pH 8.5. ScFv were passed over the immobilised protein at concentrations varying from 5-400 nM in HBS EP buffer at a flow rate of 10  $\mu$ l per min. To examine cross reactivity, the antibody was immobilised onto the surface, and S-layer proteins from *B. cereus* 11145, *B. cereus* 11143, *B. cereus* 9946, *B. thuringiensis* var. *isrealensis*, *thuringiensis* var. *kurstaki*, *B. pumilus*, *B. brevis*, *B. coagulans* and *B. atrophaeus* passed over at final concentration of 400 nM.

#### **3.45 Detection of whole spores and evaluation of sensitivity using an optical biosensor**

The resonant mirror biosensor (Thermo Labsystems, UK.) was used to demonstrate detection of whole *B. anthracis* spores. Antibodies were immobilised onto a RM T70 low molecular weight carboxymethylated dextran (CMD) cuvette surface by standard EDC/NHS coupling methods as described in. The spores were passed over at various concentrations for 10 min and the surface regenerated using 20 mM KOH for 3 min.

#### **3.46 Binding of FITC-labelled antibody to spores**

Spores were blocked with 1% BSA (w/v) for 2 h at room temperature, with mixing incubated with FITC labelled scFv in PBS 1% BSA (w/v) for 1 h at room temperature, in the dark, with mixing. The spores were washed 5 times in PBS and viewed by phase contrast and fluorescence microscopy using a. Negative controls were incorporated using FITC label and no antibody and a non-specific FITC labelled anti-ovalbumin antibody.



### **3.47 Ligation of scFv into pKappa construct**

Purified pKappa construct was kindly provided by Dr. C. Mayers (Dstl), which contained the murine light chain. Restriction digestion of the original scFv from the pAk100 plasmid and was undertaken using Sfi I (NEB). Each 50 µl reaction consisted of 2.5 µl 10x NEB #2 reaction buffer, 0.5 µl 100x BSA, 1 µl Sfi I, 1 µg of DNA to be cut, and sterile dH<sub>2</sub>O to 50µl. The restriction digests were incubated overnight at 37°C. Digestion of the pKAPPA, required for ligation of the scFv plasmid, was completed using 20 µg of vector DNA in 200 µl reaction volumes, all reagent volumes were scaled up accordingly. Crystal violet purification was carried out as described previously (section 3.38).

### **3.48 Ligation, expression and purification**

Ligation of the scFv inserts was conducted as described previously (section 3.39) using 200 ng of sfi cut plasmid and 20 ng of purified scFv DNA. The expression and purification procedure was repeated as detailed previously (sections 3.40-3.43).

### **3.49 Immunogold labelling**

#### **3.49.1 Preparation of thin sections**

The preparation of thin sections was kindly carried out by S. Smith (Dstl). Briefly, washed *B. anthracis* UM23C12 spores (10x) were centrifuged at 2000 g for 5 min and the supernatant discarded and the spore pellet resuspended in 100 µl 5% (v/v) glutaraldehyde in PIPES buffer and left to incubate for 18 h at room temperature. The spores were centrifuged again at 2 000 g for 5 min and the supernatant discarded and the pellet resuspended in 100 µl PIPES buffer (pH 6.8) and incubated for 1 h at room temperature, this wash step was then repeated. The spores were then resuspended in 50 µl 1% (w/v) low melting point agarose (Sigma) in distilled water. The agarose was allowed to set and cut into cubes (~1 mm). The samples were dehydrated by incubating twice in 100% ethanol for 30 min and then in propylene oxide for 30 min. The samples were then infiltrated with a 1:1 polypropylene/araldite mixture (from a stock comprising 175 ml Araldite resin CY212, 275 ml DDSA hardener, 5 ml dibutyl phthalate warmed to 37°C before mixing thoroughly and stored in the dark) to which 1% (v/v) BDMA accelerator was added to the appropriate volume of resin and mixed for 2 h before use. The samples were then

infiltrated with resin alone for 72 h. The samples were transferred to flat embedding moulds and polymerised at 70°C for 72 h. The blocks were left at room temperature for at least 24 h before cutting. A glass knife was used to cut ribbons of sections and floated onto pyroxylin coated nickel grids and allowed to dry. Some sections were stained and visualised by TEM prior to labelling (section 3.21.2).

### **3.49.2 Immunogold labelling**

The grids were floated on 2% (v/v) Decon 90 in sterile distilled water for 30 min at room temperature in a humid chamber. The grids were washed in sterile distilled water and dried. The grids were then floated on 50 µl drops of buffer A (PBS 0.1% (v/v) Tween 20, 1% BSA, 1% foetal calf serum, filter sterilised using a 0.2 µm filter) in a humid chamber for 30 min at room temperature. Excess buffer was blotted onto filter paper and the grid transferred to a 50 µl drop of primary antibody, diluted 1:10 scFv or 1: 100 of monoclonal antibody in buffer A, and incubated for 2 h at room temperature in a humid chamber. The grids were removed and washed on 430 µl buffer A for 5 min and the wash step was repeated. Excess buffer was blotted onto filter paper and the grids floated on a 50 µl drop of goat anti-mouse IgG 10 nm immunogold conjugate (BB international) diluted 1: 50 in buffer A and incubated for 1 h at room temperature in a humid chamber. The grids were then washed by floating on 430 µl of buffer A, twice, for 5 min then on 430 µl of distilled water. The grids were dried on filter paper and stained. Each sample was performed in duplicate and incorporated two negative controls, one with no antibody and one with a non-specific antibody. Sections were viewed under TEM (section 3.21.2).

### **3.50 Epitope mapping using linear epitopes**

The EA1 sequence was obtained from NCBI (<http://www.ncbi.nih.gov/>). The sequence was split into 14mers with 4 amino acid overlap either side. The sequences were synthesised by Alta Bioscience (Birmingham, UK). The scFv were immobilised onto a CM5 sensor surface using standard EDC/NHS coupling methods as described previously (3.25.5.1). Peptides were passed over the surface for 10 min and the surface regenerated using 20 mM KOH as described previously (section 3.25.5.2).

## Chapter 4 Disruption of bacterial endospores

### 4.1 Introduction

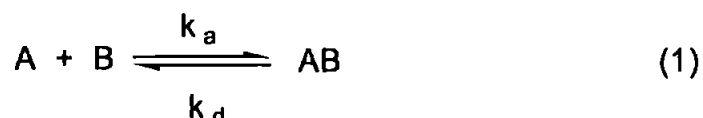
There is a requirement for rapid detection of *B. anthracis* spores in order to administer effective treatment to a susceptible population. Therefore, both the specific assay and sample processing prior to this must ensure that the pathogen can be rapidly detected (Webb, 2003). One detection technology that has demonstrated real time detection of a range of smaller molecular weight analytes is the optical evanescent biosensor. This type of biosensor is based on the properties of evanescent wave principles and uses small changes in optical properties to directly monitor these changes. The use of these biosensors is particularly attractive as they provide a means of real time continuous monitoring for the presence of pathogenic microorganisms compared to standard techniques (Ghindilis *et al.*, 1998; Gizeli and Lowe, 1996; Braguglia, 1998; Cruz *et al.*, 2002). However, this application has been limited due to the relative insensitivity of the assay for detection of bacteria compared to standard methodologies (Watts *et al.*, 1994; Framatico *et al.*, 1998).

The optical biosensor chosen for this work was the resonant mirror. This instrument detects changes in the refractive index that occur near the sensor surface, where the change in the refractive index is dependent on the mass of material bound to the surface. As analyte binds to surface immobilised ligand, the response is measured directly without any requirement for the addition of secondary reagents. The size of the response is dependent on  $\Delta n$ , defined as the difference between the refractive index of the material bound and that of the water displaced (1.33). A large response is observed on protein binding as the refractive index is approximately 1.51 thus,  $\Delta n = 0.18$ . Bacterial spores are highly refractile due the dehydrated nature of the core and the refractive index of a dormant bacterial spore is close to that of a fully dried protein with  $\Delta n = 1.52$  (Tuminello *et al.*, 1997; Katz *et al.*, 2005). However, detection of bacterial spores using optical evanescent biosensors has proved particularly difficult (Dstl, Detection Department, unpublished data). The reason for the relatively high detection limits of bacterial spores despite their higher refractive index is unclear.

To produce a specific detection system using optical evanescent sensors appropriate ligands, such as antibodies, must be chemically immobilised onto a sensor surface. Thus, a

further consideration is the availability of ligand to bind to analyte. Dextran coated sensor surfaces have been widely used for optimum protein detection, since this allows for higher density of antibody to be immobilised and for reduced non-specific binding compared to other surfaces. The dextran matrix is greater than 100 nm thick, and antibodies immobilised within this layer are easily accessible to large protein molecules such as toxins. However, intact bacterial cells or spores are not able to penetrate it and only antibody on the surface of the gel would be accessible. Thus, on binding a large proportion of the spore, typically 0.5-1  $\mu\text{m}$  would be outside of the evanescent field which has been suggested to reduce assay sensitivity for bacterial cells (Watts *et al.*, 1994; Framtico *et al.*, 1998; Horváth *et al.*, 2003). The large size of the bacterial spore in comparison to proteins produces further restrictions on detection. In addition, it may not be possible for the immobilised ligands to come into contact with epitopes on the spore due to steric hindrance, limiting binding events.

Reduction in the rates of mass transport of larger particles when using optical biosensors is another effect that reduces the sensitivity of bacterial detection (Leatherbarrow and Edwards, 1999). The reaction of analyte in solution A with the immobilised antibody B to form the antibody-analyte complex AB is governed by the equilibrium



and the rate is given by the formula:

$$d[AB]/dt = k_a[A][B] - k_d[AB] \quad (2)$$

where  $k_a$  is the association rate constant and  $k_d$  is the dissociation rate constant. The rate of response of the detector is therefore proportional to the concentration of the analyte in solution. Using the resonant mirror, to obtain a significant response to the binding of a protein of molecular weight 60 kDa, in a solution of 50 ng/ml, the concentration would be 0.8 nM or  $4.8 \times 10^{11}$  molecules/ml. The desired lowest detectable concentration for larger particles such as spores would be many orders of magnitude below this (Fox *et al.*, 2002; Ivnitcki *et al.*, 1999). Although the binding reaction for bacterial spores would be more

complex due to the presence of multiple copies of a protein on the surface, theoretically the lower concentration of analyte means that the rate of binding would be considerably less than soluble protein. The binding of bacterial spores is also likely to be limited by mass transport effects, being dependent on the diffusion coefficient,  $D$ . Larger particles such as bacterial endospores would show reduced mass transport to the sensor surface compared to the smaller protein molecules, again reducing the rate of binding. Thus, disruption of cells or endospores should enhance assay sensitivity both by increasing the concentration of protein available for binding and by reducing mass transport limitations.

There are a range of methods that can be employed to achieve disruption of bacterial cells. However, bacterial spores are inherently resistant to mechanical, enzymatic and chemical methods of disruption, therefore the development of a rapid disruption step is a considerable challenge. The addition of enzymes and chemicals and other treatments such as high pressure have been reported to achieve physical disruption using milder conditions (Hamilton and Sale, 1967; Simpson *et al.*, 1995; Raso *et al.* 1998; Pol *et al.*, 2000; Luna *et al.*, 2003). Once spores have germinated they are less resistant to disruption than those in the dormant phase. A range of conditions have been found to induce germination including cationic surfactants, the addition of  $\text{Ca}^{2+}$  dipicolinic acid (DPA) complex and exposure of the spores to high pressures (Setlow, 2003). However, the induction of germination would increase the processing time as a germinant will require time to take effect (Hamilton and Sale, 1967). Thus, the use of enzymes and chemicals were not deemed suitable for rapid detection methods.

Kuske *et al.* (1998) examined the use of hot-detergent treatment, freeze-thaw cycles and bead mill homogenisation to break spores to allow for DNA extraction. Bead mill homogenisation was reported as being a successful technique for DNA release but was discounted for use in the present study because it would be impractical in a rapid detection system, due to the requirement for glass beads and multiple passes. Nebulisation was also discounted due to the need for multiple treatments. (Jensen *et al.*, 1992; Agranovski *et al.*, 2002). Although there are conflicting reports of the utility of freeze thawing for the disruption of bacterial spores (Tsai and Olsen, 1991; Carl *et al.*, 1992; Herrick *et al.*, 1993; Picard *et al.*, 1992; Moré *et al.*, 1994; Kuske *et al.*, 1998) this method would obviously not be practicable for a rapid in-line disruption system.

Sonication is generally achieved by the rapid vibration of the sonicator probe tip producing high intensity sound waves, which induces the formation of cavities or gas nuclei in the aqueous phase. The bubbles formed may either oscillate, sometimes referred to as stable cavitation, or implode, often termed transient cavitation (Leighton, 1994; Segebarth and Reisse, 2002). The process itself is thought to occur through particulates contained within liquid medium acting as bubble nuclei. As a number of bubbles interact with each other, a cavitation field is produced (Suslick *et al.*, 1999). In the case of transient cavitation, micro-streaming can occur creating large shear forces, particularly at a phase boundary, such as the gas liquid phase boundary of a bubble. These shear forces may be large enough to induce damage on the surface of biological moieties. In the case of transient cavitation, bubbles undergo explosive growth followed by rapid collapse, thus, a shock wave is generated thought to be sufficient to lyse cell walls (Hughes and Nyborg, 1962; Leighton, 1994; Huang *et al.*, 2002). When the bubbles implode, the diffuse sound energy is concentrated into localised spots as the compression of the gas contained within the bubble generates heat. This occurs more rapidly than thermal diffusion, creating short lived localised areas of high temperature and pressure, as well as high rates of heating and cooling (Suslick *et al.*, 1999). The conditions achieved during this collapse can also drive chemical reactions. For example, free radicals can form from sonication of water. In this case,  $\cdot\text{OH}$  and  $\cdot\text{H}$  radicals are produced through the homolysis of water molecules. These may then reform as water ( $\text{H}_2\text{O}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) or hydrogen ( $\text{H}_2$ ) (vonn Sonntag *et al.*, 1999). The majority of work undertaken for biological systems has examined the sonication of cells or spores to release internal components, such as DNA. Although the breakage of bacterial cells is relatively easy, spores have proven more problematic due to their high resistance to mechanical breakage even over prolonged time periods of exposure to cavitation (Berger and Marr, 1960). The main disadvantages of the technique is the potential for aerosol production within some systems, prolonged treatment time and the possible need for ear protection for the operator. Advantages include a possible reduction in the clumping and the release of soluble antigen from the spore surface improving detection, even without spore breakage.

Electroporation is another method that has been examined for cellular disruption, although the mechanism is still not fully understood. Electroporation occurs when strong electric pulses are applied to a membrane, causing a significant increase in conductivity and permeability (Lebar *et al.*, 2000). In the electroporation of a bacterial cell the main features

of the process are the formation of pores within a lipid membrane on exposure to high electric fields (Castro *et al.*, 1993; Vega-Mercado, 1996). If the size and number of the pores formed becomes large compared to the surface area of the membrane then irreversible damage occurs leading to cell death (Zimmerman, 1986). A model of the effects of electric fields on bacterial membranes has been described by Zimmerman (1986) However, the target of electroporation within the bacterial spore, having a very different structure to that of a cell is unclear and reports of the electroporation of bacterial spores are scarce, show large variance in results and are inconclusive (Hamilton and Sale, 1967; Knorr *et al.*, 1994; Marquez *et al.*, 1994; Simpson *et al.*, 1995).

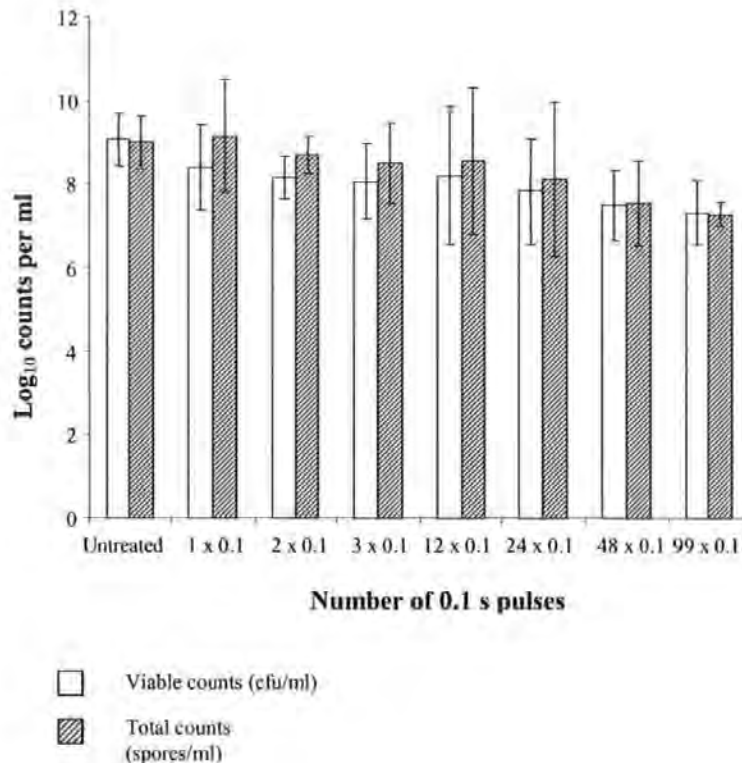
The aim of this work was to examine sample processing prior to the detection assay. Disruption techniques have been evaluated and characterised to enhance the sensitivity of bacterial detection, whilst trying to maintain the speed of the assay and minimise the requirement for any additional reagents. Electroporation and sonication were evaluated and examined for their potential for rapid disruption of bacterial spores. In preliminary experiments *B. atrophaeus* was used as a model organism for *B. anthracis* to characterise the disruption techniques. *B. atrophaeus* is used widely as a model for *B. anthracis* due to ease of manipulation and its use within the field to evaluate detection technologies. The knowledge gained for the development of an in-line disruption method for improved rapid immunological based detection of *B. anthracis* spores and could be used in further work for the identification of suitable detection targets and the rational design of specific ligands.

## 4.2 Results

In the following descriptions, 'breakage' is used to define the complete fracturing of cell or spores to release internal components. The term 'disruption' is used to define any alteration of the exposed surface of the cell or spore. Viable counts represent the number of organisms capable of growth following incubation on LB agar overnight at 37°C (section 3.12) and total counts represent the number of intact organisms, calculated as detailed in section 3.20

#### 4.2.1 Examination of the effects of electroporation

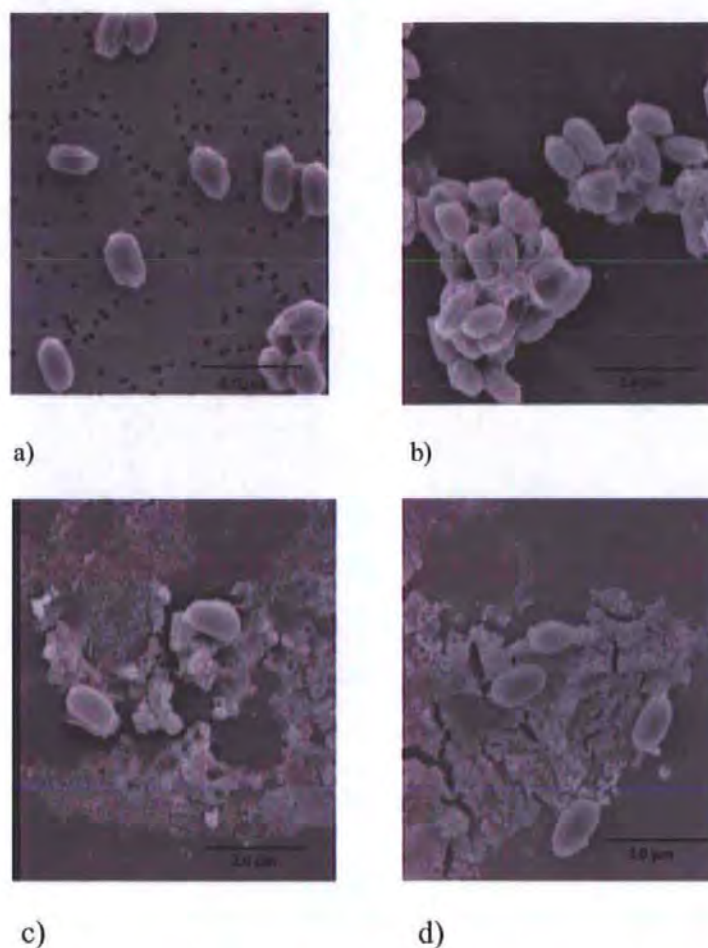
In order to determine the effect of electroporation on the spore, a preliminary analysis of the extent of spore breakage was undertaken using total and viable counts. The statistical significance of the results was determined using the analysis of variance (ANOVA) assuming  $P < 0.05$ . Figure 4.1 shows that increasing the duration of exposure to the electric field resulted in a decrease in the total and viable counts (total  $P = 7.528 \times 10^{-17}$ ; viable  $P = 8.803 \times 10^{-17}$ ). However, differences were not significant for shorter periods of exposure and no significant increase in disruption was observed above 12 pulses. Even after electroporation for 99 pulses, the reduction in the cell counts was only approximately 10-fold.



**Figure 4.1 Assessment of the disruption of *B. atrophaeus* spores by electroporation for increasing numbers of 0.1 s pulses by total and viable counts.** Washed *B. atrophaeus* spores (at  $1 \times 10^9$  cfu/ml) were electroporated at 5 kV/cm using a square wave pulse of 0.1 s width, increasing the number of pulses. Error bars show  $\pm 2$  standard deviations from the mean.



As spore breakage was limited, the effects of the application of an electric field on the outer spore surface was examined by scanning electron microscopy (SEM). This showed that untreated spores had characteristic ridges on the surface. When spores were exposed to twelve short square wave pulses of duration of 0.0001 s at a higher field strength (7.5 kV/cm) no observable debris resulted and the sample looked very similar to the untreated control (Figure 4.2a). However, with the application of the lower field strength (5.0 kV/cm) but longer single pulse duration (0.1 s) some debris was observed. It was also noted that although most spores remained intact, some had an abnormal shape, characterised by a lack of ridging on the spore coat and/or pustules on the surface. Similar results were obtained with *B. atrophaeus*, *B. anthracis* UM23C12 and *B. anthracis* Sterne; representative results are shown in Figure 4.2.

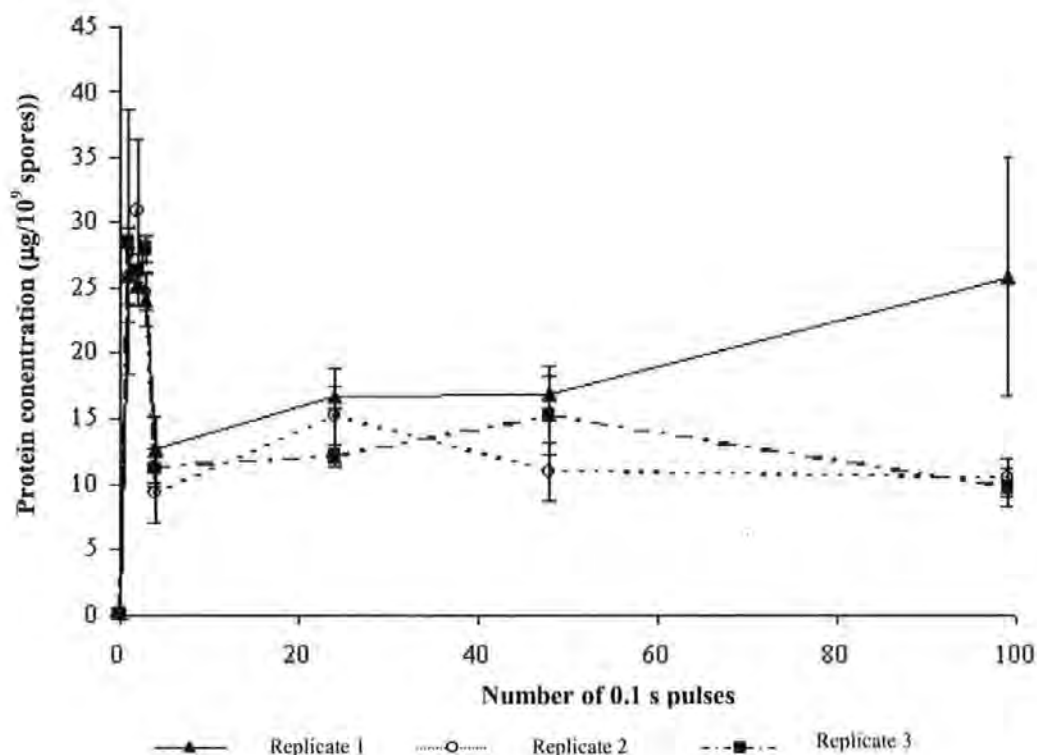


**Figure 4.2** SEMs showing electroporation of *B. anthracis* UM23Cl2 spores for increasing numbers of 0.1 s pulses. Washed *B. atrophaeus* spores (at  $1 \times 10^9$  cfu/ml) were electroporated at 7.5 kV/cm or 5 kV/cm using a square wave pulse of 0.1 s width, increasing the number of pulses. a) untreated control b) 7.5 kV/cm 12 x 0.0001 s, c) 5 kV/cm 12 x 0.1 s, d) 5 kV/cm 24 x 0.1 s .

#### 4.2.1.1 Evaluation of protein release

The concentration of protein released from  $1 \times 10^9$  spores/ml *B. atrophaeus* spores by electroporation was analysed by BCA protein assay and SDS PAGE. The concentration of protein released by electroporation at 7.5 kV/cm for 12 x 0.0001 s pulses gave a protein concentration of  $\sim 95$   $\mu\text{g/ml}$  by the BCA assay. Results were difficult to interpret due to large differences observed in the amount of protein released under the same conditions for each replicate, shown and a relationship between the protein released and time period of electroporation was difficult to ascertain. Repeated replication of the experiment showed that there was a large spread in the results, but that the amount of protein detected

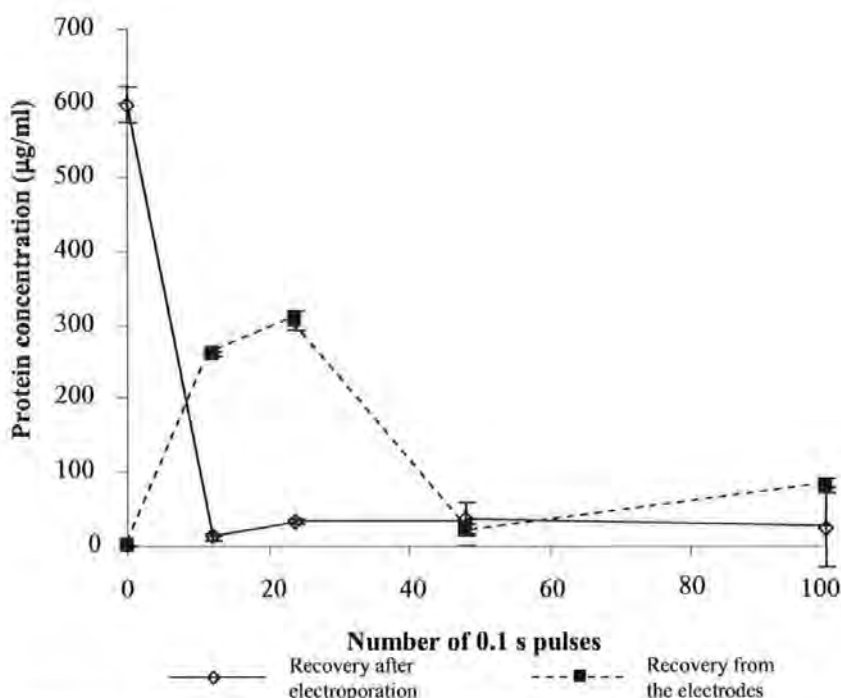
decreased with an increasing number of cycles of electroporation (Figure 4.3). These results were supported by SDS-PAGE (not shown).



**Figure 4.3 Protein release from *B. atrophaeus* spores electroporated for increasing numbers of 0.1 s pulses.** Washed *B. atrophaeus* spores (at  $1 \times 10^9$  cfu/ml) were electroporated at 5 kV/cm using a square wave pulse of 0.1 s width, increasing the number of pulses. The results show three batches of data, denoted as replicates, 1, 2 and 3. Error bars show  $\pm 2$  standard deviations from the mean.

The reason for this decline was difficult to determine in a complex protein suspension; therefore the effects of electroporation on an individual protein was investigated. Ovalbumin (500  $\mu\text{g/ml}$ ) was electroporated in the same way as *B. atrophaeus* spores. Electroporation of protein revealed a decrease in concentration over increasing time periods (Figure 4.4). To investigate the possibility that the protein was either being denatured and precipitating, or was forming deposits on the electrodes a further assay using detergents to wash out the cuvette after electroporation was applied. This showed that

some proteins were removed from the electrodes (Figure 4.4). However, this did not account for all the protein present before electroporation, suggesting that either a proportion of the protein may have been removed from the solution within the electroporation cuvette and deposited on the electrodes and/or the proteins were irreversibly denatured and had precipitated.



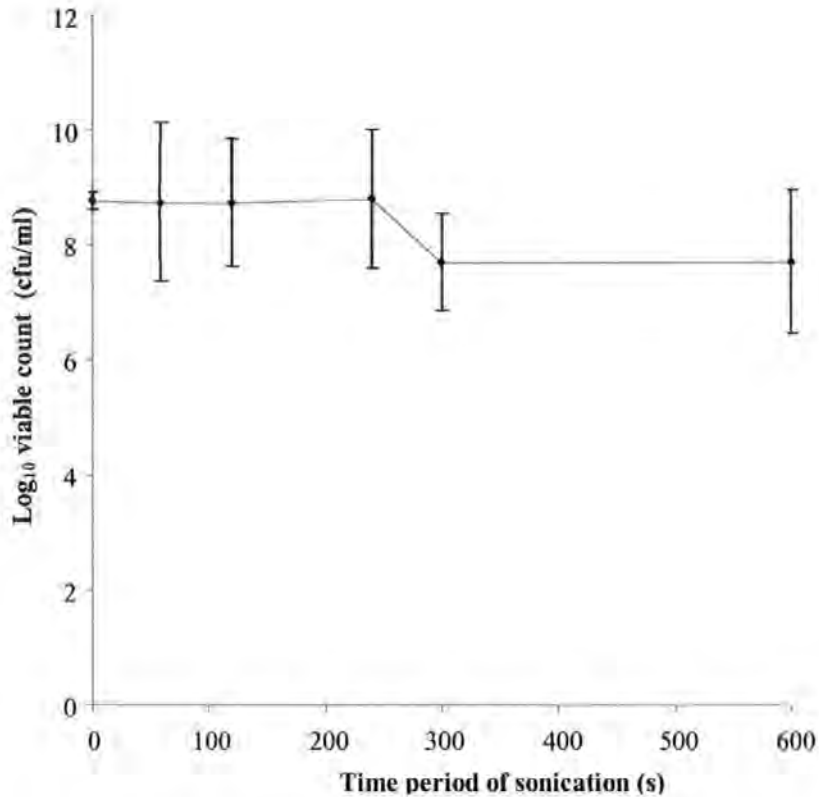
**Figure 4.4 Recovery of ovalbumin after electroporation for increasing numbers of 0.1 s pulses.** Ovalbumin (at 500 µg/ml) was electroporated at 5 kV/cm using a square wave pulse of 0.1 s width, increasing the number of pulses. The concentration recovered after electroporation and that recovered through extensive washing of the electrodes with PBS Tween 20 0.05% was assessed. Error bars show  $\pm 2$  standard deviations from the mean.

In order to determine if the spores were being broken by electroporation or only disrupted and spore proteins released a number of experiments were conducted. The first of these was to determine if any DNA was released following electroporation. The polymerase chain reaction (PCR) was used to detect any DNA released. However, no positive results were obtained in PCR assays after the electroporation of washed *B. atrophaeus* spores. To assess if electroporation was having an effect on the DNA alone purified *B. atrophaeus*

DNA was also subject to the same conditions as had been used for whole spores. The DNA was not detectable by PCR following electroporation, suggesting that the electric field did effect the DNA and thus this method was not pursued as a measure of spore breakage. Assessment of the release of DPA to determine spore breakage was therefore conducted and compared to sonication and is detailed in section 4.2.2.2. Analysis of the release of surface proteins released was conducted by separation of the proteins by SDS-PAGE and detection of those recognised by antibody determined by Western blot. This was also compared to sonication and is detailed in section 4.2.2.1. The detection of any spore antigen released by sonication was completed using the resonant mirror biosensor. In this case a polyclonal rabbit anti-*B. atrophaeus* antibody was immobilised onto a sensor surface and electroporated spores introduced. It was not possible to detect untreated or electroporated spores using the resonant mirror biosensor, thus this method was unsuitable for enhancement of assay sensitivity using the conditions tested and was not pursued further.

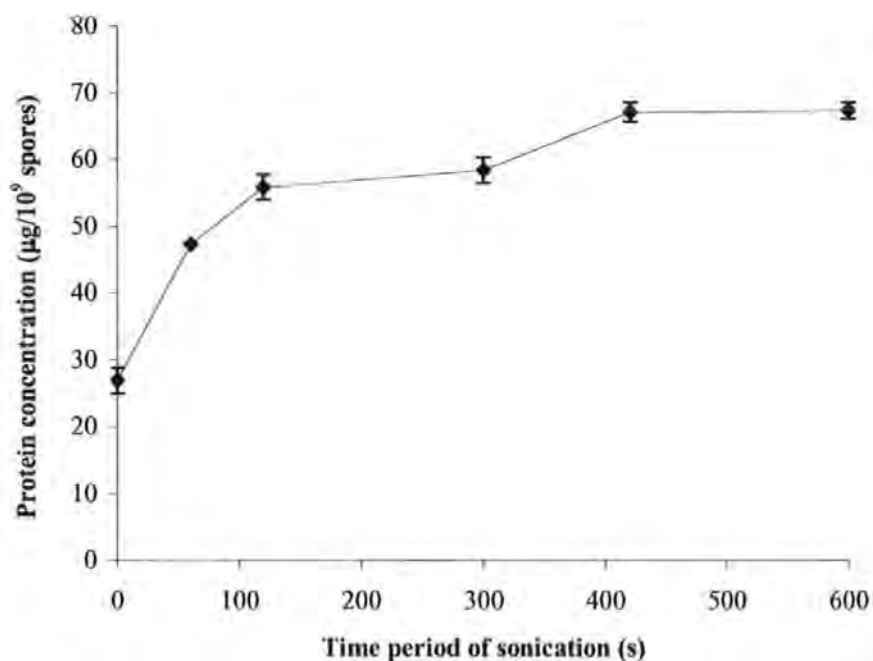
#### **4.2.2 Evaluation of a standard probe sonicator**

Initial investigations of the effects of sonication on bacterial spores were undertaken using a probe sonicator (Misonix). The statistical significance of the results was determined using the analysis of variance (ANOVA) assuming  $P < 0.05$  ( $P = 0.892$ ). There was no statistically significant effect of up to 600 s sonication on the viability of *B. atrophaeus* spores (Figure 4.5).



**Figure 4.5** Viable counts of *B. atrophaeus* spores after sonication with the standard probe sonicator for varying time periods. *B. atrophaeus* spores were washed and sonicated at approximately  $1 \times 10^9$  cfu/ml using a standard probe sonicator (Misonix) at 40% power in 10 s pulses for varying time periods. Error bars show  $\pm 2$  standard deviations from the mean

Increasing time periods of sonication up to 120 s had a significant effect on the release of protein (Figure 4.6). The maximum amount of protein released was approximately 65  $\mu\text{g/ml}$  after 7 min of sonication of  $1 \times 10^9$  *B. atrophaeus* spores/ml. However, it was not possible to detect treated or untreated spores using the resonant mirror biosensor.

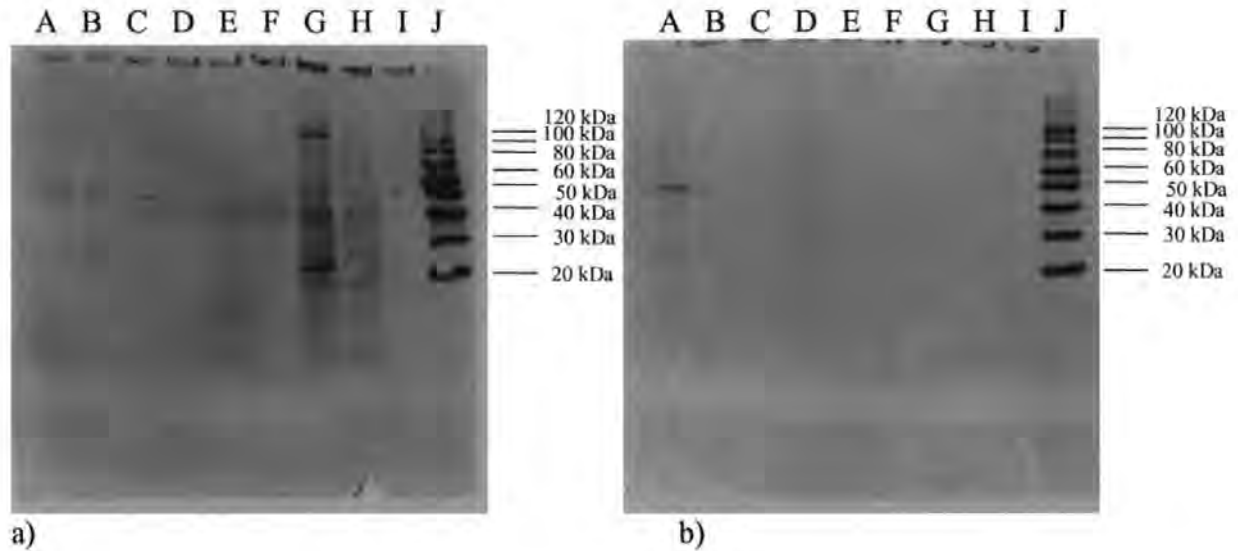


**Figure 4.6 Protein assay of *B. atrophaeus* spore proteins sonicated for various time periods.** *B. atrophaeus* spores were washed and sonicated at  $1 \times 10^9$  cfu/ml using a standard probe sonicator (Misonix) at 40% power in 10 s pulses for varying time periods. The concentration of protein released was determined by BCA assay. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.2.1 *Detection of the release surface proteins by electroporation and sonication by western blot analysis*

As electroporation and sonication both released protein that was not detectable by antibody, the release of surface proteins from the spores was determined. Spores were biotinylated using sulfo-NHS-biotin (Pierce), the sulphate group allowing for solubility at physiological pH and labelling surface located proteins. Following sonication or electroporation of the spores, the supernatants containing the proteins released were separated by SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen), blotted onto PVDF membranes.





**Figure 4.7** Western blot analysis of the detection of the release of biotinylated surface proteins from *B. atrophaeus* spores by sonication and electroporation. Following sonication using a standard probe sonicator the proteins release were separated by SDS-PAGE on 4-12% Bis-Tris precast gels (Invitrogen). The proteins were then transferred to a PVDF membrane and probed with a streptavidin peroxidase conjugate (1:1000). Biotinylated proteins were visualised by ECL™ detection (Amersham). Lane loading was as follows: a) Sonicated *B. atrophaeus* spores A) 5 s, B) 30 s, C) 60 s, D) 120 s, E) 240 s, F) 300 s, G) 420 s, H) 600 s., I) untreated control (biotinylated no sonication) J) Magic Mark™ Western Standard. b) Electroporated *B. atrophaeus* spores. A) 5 kV/cm 1 x 0.1 s, B) 5 kV/cm 2 x 0.1s, C) 5 kV/cm 3 x 0.1s, D) 5 kV/cm, 12 x 0.1. E) 24 x 0.1s 5 kV/cm, F) 5 kV/cm 48 x 0.1s. G) 5 kV/cm 99 x 0.1s, H) 99 x 0.1s wash. I) biotinylated no electroporation. J) Magic Mark™ Western Standard

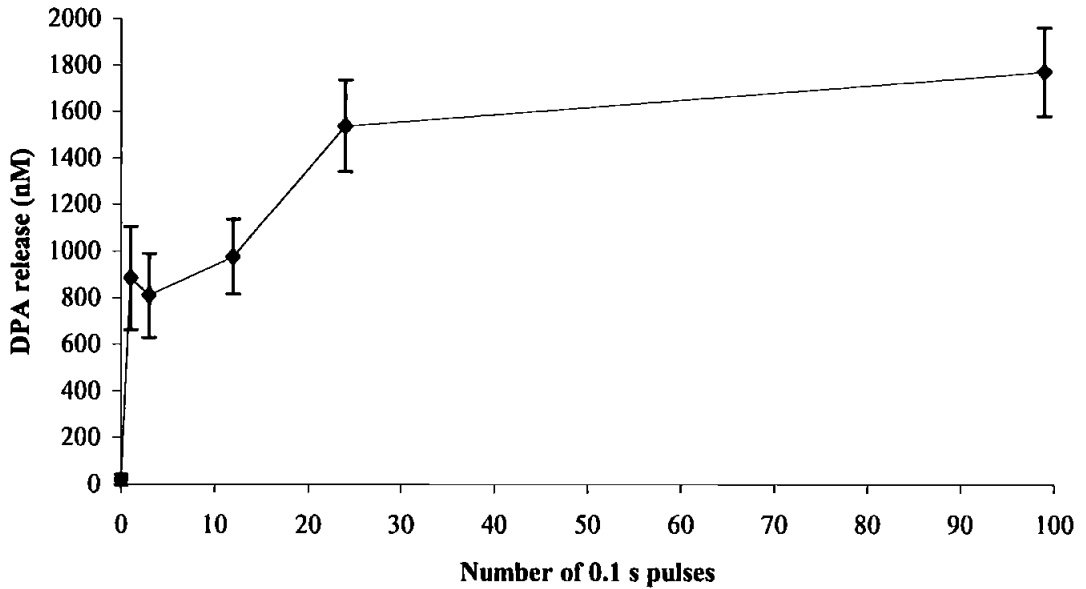
Western blot analysis of biotinylated surface proteins released from sonicated or electroporated samples revealed a distinct difference between the two samples. In the case of sonication, increasing amounts of proteins were detectable as the time period of the sonication increased, reaching a maximum after 7 min (Figure 4.7a). There appeared to be two predominant surface proteins released by the sonication process (40 and 45 kDa). The apparent protein concentration and protein profile changed above 420 s most notably with



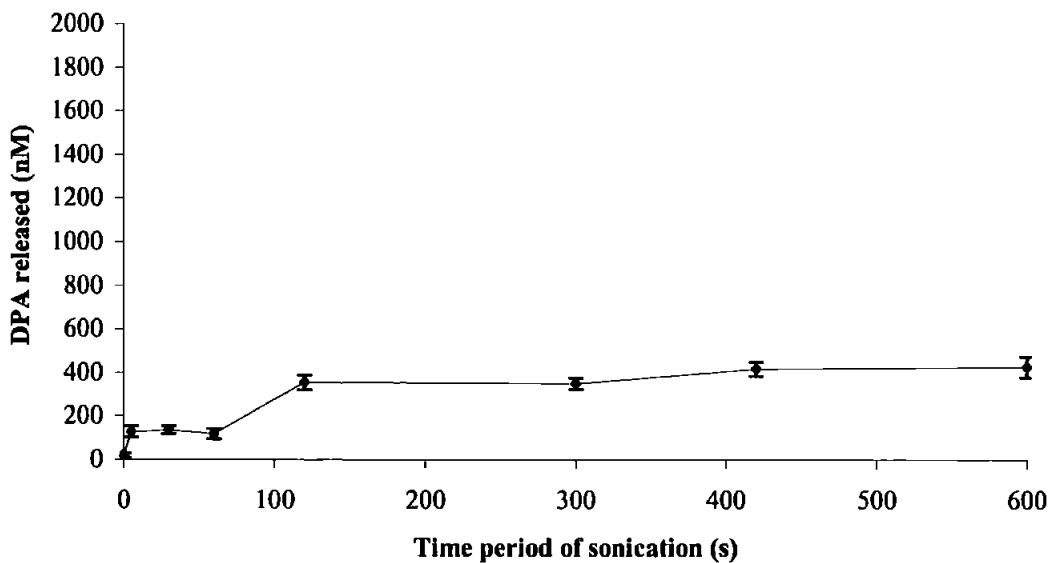
the apparent loss of the 45 kDa protein. In the case of the electroporated sample, biotinylated proteins were only detected after the shortest (1 x 0.1 s) electroporation period (Figure 4.7b). In both cases, there was no binding of the streptavidin peroxidase conjugate to the non-biotinylated and electroporated or sonicated controls completed for each time period of each treatment (data not shown).

#### ***4.2.2.2 DPA release from spores***

Following sonication, the concentration of DPA was determined using a method based on that described by Hindle and Hall (1999). Increasing periods of electroporation or sonication resulted in a similar pattern of DPA release, although the amount released was approximately 4.5 times higher using electroporation (Figures 4.8a) compared to sonication (Figure 4.8b).



a)

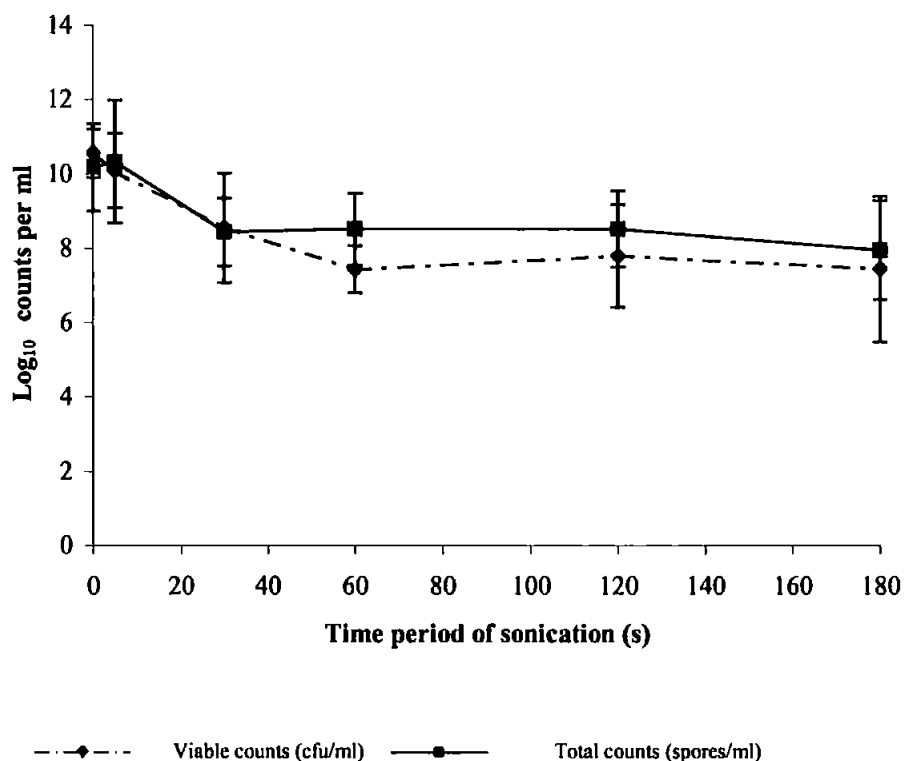


b)

**Figure 4.8 DPA release from *B. atrophaeus* spores a) electroporated for increasing numbers of 0.1 s pulses and b) sonicated for increasing time periods.** Washed *B. atrophaeus* spores, at  $1 \times 10^9$  cfu/ml, were sonicated at 40% power using a standard probe sonicator (Misonix) or electroporated at  $5 \text{ kV/cm}^{-1}$  using a square wave pulse of 0.1 s width, increasing the number of pulses. Error bars show  $\pm 2$  standard deviations from the mean.

### 4.2.3 Evaluation of the extent of disruption and effects of sonication using the Cepheid minisonicator system

The Cepheid minisonicator was the second system evaluated as previous work using PCR suggested that using this system spore breakage was achieved after 30 s (Belgrader *et al.*, 1999). Therefore, this system was evaluated for its utility for the improvement of immunoassay sensitivity. Initial investigations of the extent of spore breakage were conducted using total and viable counts. The statistical significance of the results was determined using the analysis of variance (ANOVA) assuming  $P < 0.05$ . Figure 4.9 shows the there was an effect of increasing periods of sonication on the total and viable counts of *B. atrophaeus* spores (viable,  $P = 1.845 \times 10^{-17}$ ; total,  $P = 1.905 \times 10^{-17}$ ). Viable and total counts showed very similar trends. No significant reduction in counts was observed with less than 30 s sonication (for 30 s viable  $p = 1.092 \times 10^{-7}$ ; total  $p = 1.462 \times 10^{-7}$ ) but times above 60 s did not result in further loss.



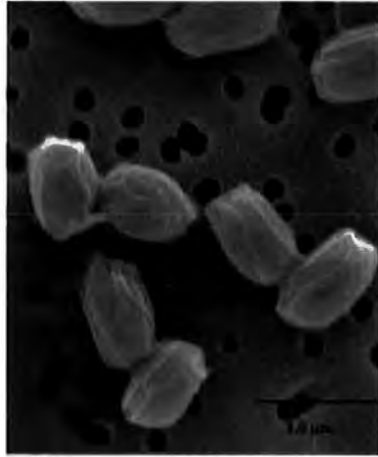
**Figure 4.9** A comparison of total and viable counts of *B. atrophaeus* spores after sonication for various time periods. The spores were washed and sonicated using the Cepheid system at 100% amplitude for defined time periods. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.3.1 *Electron Microscopy*

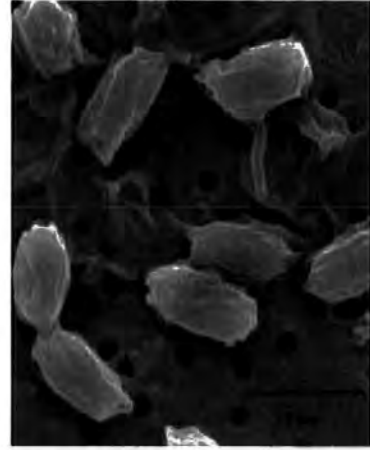
The effects of Cepheid sonication on the structure of *B. atrophaeus* spores were observed by SEM. Untreated samples revealed that the outer spore coat showed ridges running parallel to the axis of the spore (Figure 4.10a) There was some evidence of ghost cells and some debris, but this was not extensive. After a sonication period of 5 s, there was greater evidence of ghost cells (Figure 4.10b). The outer spore coat also appeared rougher than the untreated sample. There was some evidence of the appearance of small fragments after 30 s sonication (Figure 4.10c). After sonication for 120 s, many spores were broken open and large quantities of debris were observed, although some spores did appear to remain intact (Figure 4.10d).

Figure 4.11 shows the effect of sonication on the appearance of *B. anthracis* spores by SEM. After 30 s sonication there was some evidence of disruption to the outer layers of the spore and fracturing of the spore coat observed as a line or crack running down the axis of the spore (Figure 4.11b). After 120 s sonication a significant amount of disruption and breakage was observed (Figure 4.11c and 4.11d) which was accompanied by a large amount of debris, representing spore fragments and possible aggregates of precipitated protein, although the effect was not homogeneous throughout the sample (Figure 4.11d).

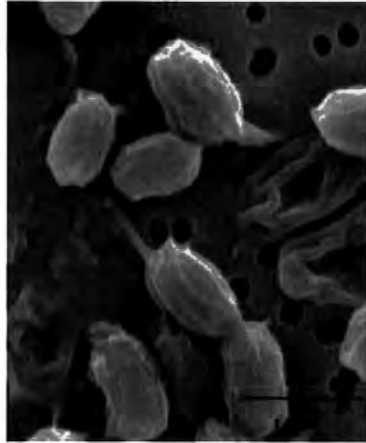
The extent of disruption of *B. anthracis* spores was also observed by transmission electron microscopy (TEM). Figure 4.12a shows the untreated control with distinct structures clearly visible. Figure 4.12d shows that sonication resulted in removal of the exosporial layers and fracture of the spore coat and all inner structures to release core components. However, Figure 4.12b demonstrated that exosporium removal and spore breakage does not occur in a uniform manner for a given sample. However, there was apparent enlargement and darkening of the core and surrounding cortex.



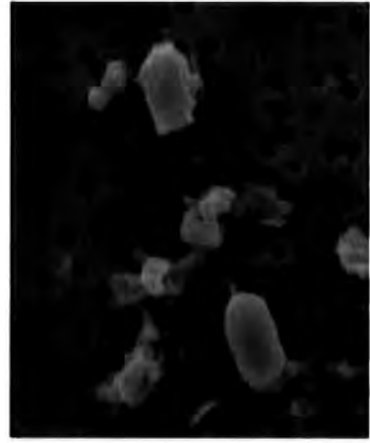
a)



b)



c)



d)

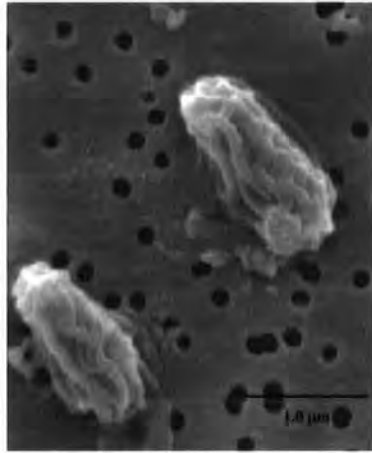
**Figure 4.10** SEMs of *B. atrophaeus* spores a) untreated and b)-d) sonicated using the Cepheid system at 100% power b) 5 s, c) 30 s, d) 120 s.



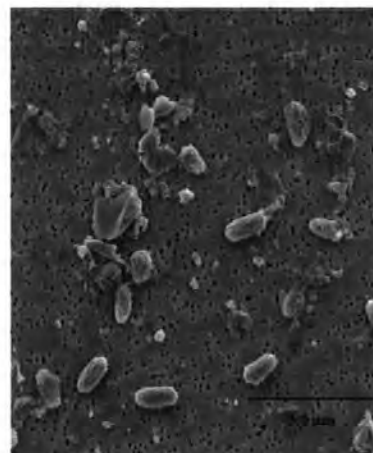
a)



b)

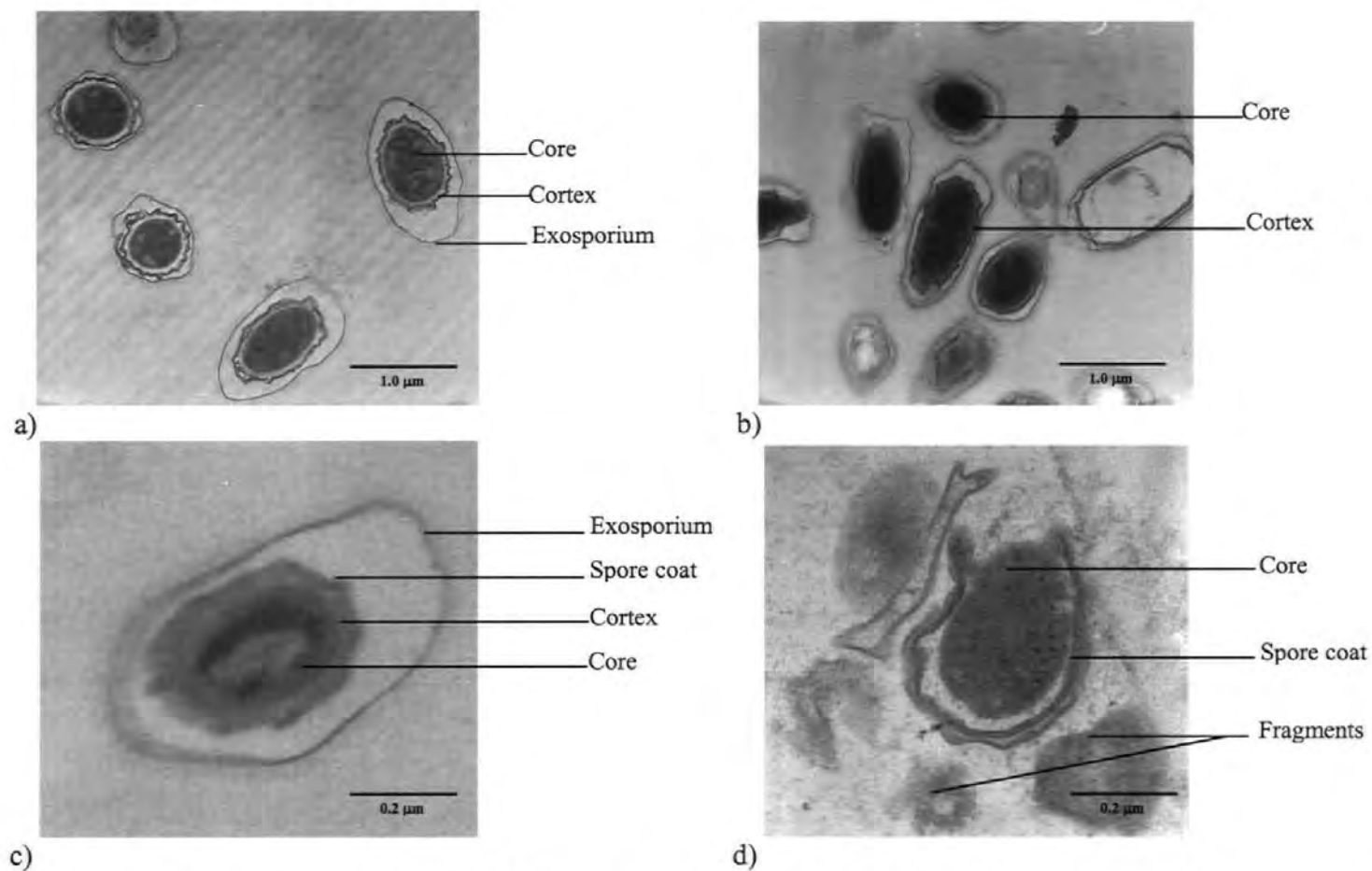


c)



d)

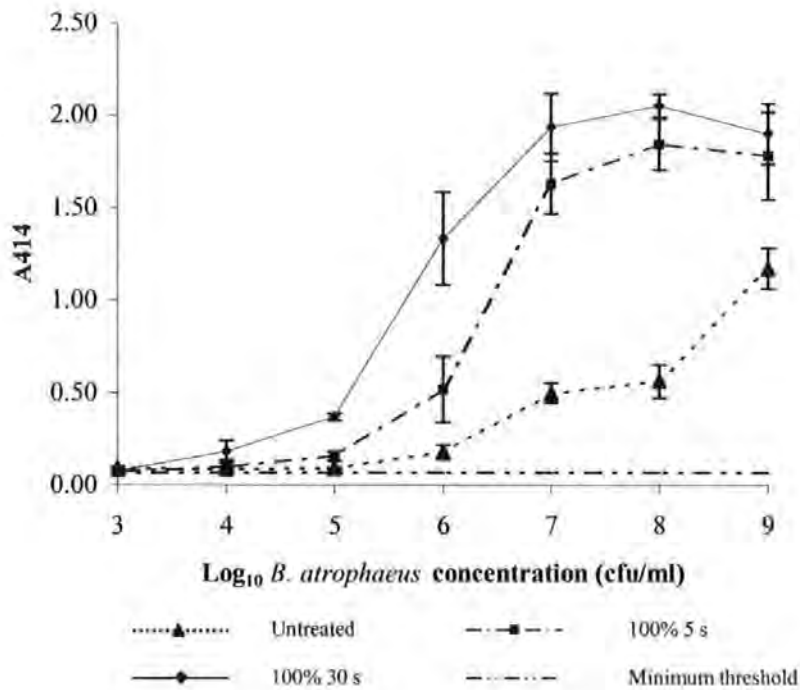
**Figure 4.11 SEMs of *B. anthracis* spores ( $\gamma$ -irradiated vaccine strain) sonicated using the Cepheid system at 100% power for a) untreated, b) 30 s c) 120 s, d) 120 s.**



**Figure 4.12** TEMs of *B. anthracis* UM23C12 spores sonicated using the Cepheid system. Washed spores were sonicated for defined time periods using the Cepheid system at 100% power a) Untreated *B. anthracis*  $\gamma$ -irradiated vaccine strain b) sonicated (120 s) *B. anthracis*  $\gamma$ -irradiated vaccine strain, c) Untreated *B. anthracis* UM23C12, d) sonicated (120 s) *B. anthracis* UM23C12

#### 4.2.3.2 Assessment of the effects of sonication by ELISA

Preliminary investigation of the effects of disruption on an immunoassay based technique was conducted by ELISA. For comparative analysis of the results the lowest detectable concentration (i.e. that giving a positive result) was defined as any value above the background + 2 standard deviation of the mean (termed the minimum threshold). By direct ELISA it can be seen that there was an approximate 10 fold increase in assay sensitivity after sonication of *B. atrophaeus* spores for 5 s, and an approximate 100 fold increase after sonication for 30 s (Figure 4.13).



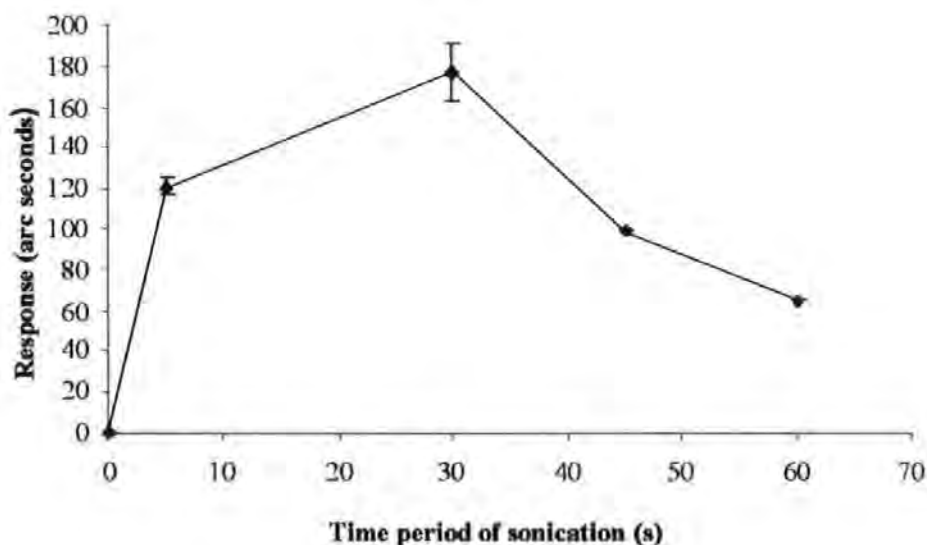
**Figure 4.13** Detection of *B. atrophaeus* spores varying the time period of Cepheid sonication (100% amplitude) by direct ELISA. The antibody used was a polyclonal rabbit anti-*B. atrophaeus* antibody (Dstl.). Error bars show  $\pm 2$  standard deviations from the mean.



#### 4.2.3.3 Assessment of the effects of Cepheid sonication using the resonant mirror

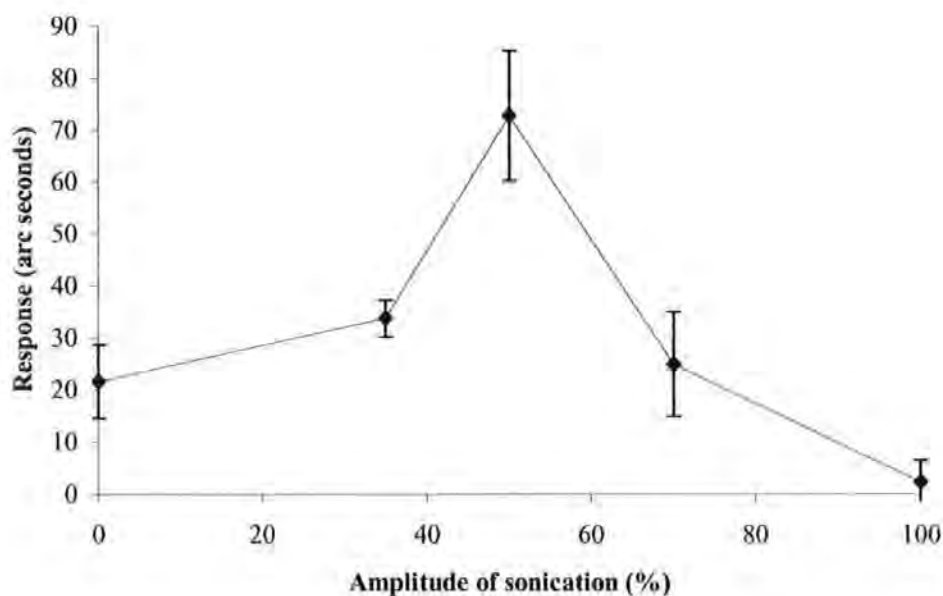
The resonant mirror was used as a near real-time detection technology to analyse the effects of sonication at various amplitudes and for varying time periods on antigen release and any deleterious effects of the sonication process.

The analysis of the detection of an analyte using the resonant mirror biosensor are described by Lowe *et al.* (1997). Preliminary experiments showed that the optimum amplitude for sonication was 100%. However, although the magnitude of the response for each detectable concentration of spores tested increased with increasing amplitude of sonication, there was only a slight improvement in the assay sensitivity (data not shown). Figure 4.14 shows that untreated *B. atrophaeus* spores were not detectable using the resonant mirror biosensor. Sonication for 5 or 30 s resulted in a significant increase in assay sensitivity, but sonication of *B. atrophaeus* spores for longer than 30 s demonstrated a significant decrease in the sensitivity of the assay.



**Figure 4.14** Degradation in the response of the resonant mirror biosensor to the detection of  $1 \times 10^8$  cfu/ml *B. atrophaeus* spores varying the time period of Cepheid sonication at 100% amplitude. The antibody used was a polyclonal anti-*B. atrophaeus* antibody (Dstl) which was immobilised onto a low molecular weight (T70) carboxymethylated dextran cuvette. Error bars show  $\pm 2$  standard deviations from the mean.

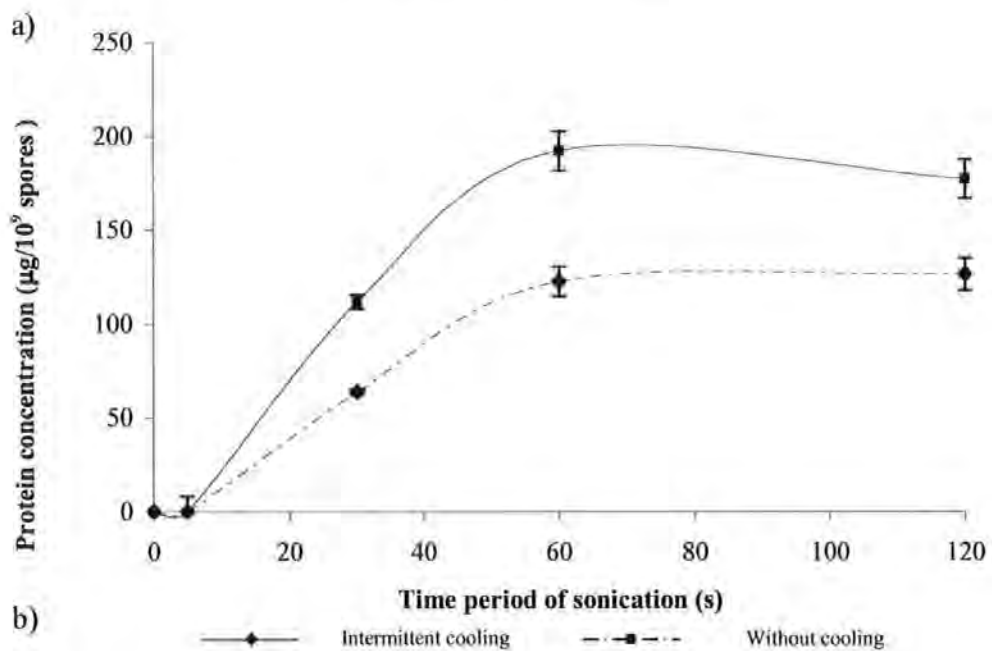
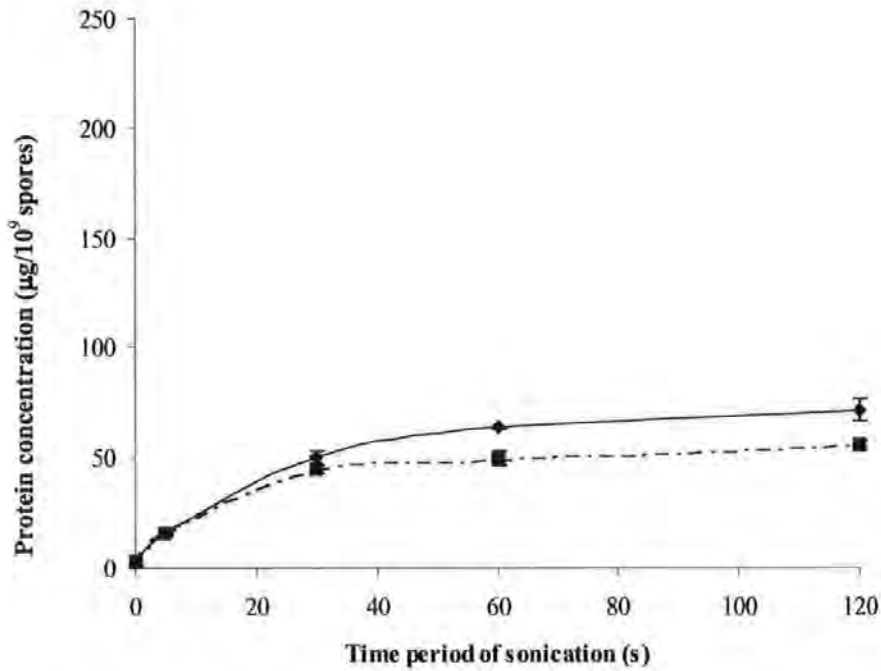
For *B. anthracis* UM23C12 spores, untreated spores were detectable using the resonant mirror biosensor at  $1 \times 10^8$  spores/ml. Preliminary experiments showed that the optimum amplitude for sonication appeared to be 50% for 5 s; at amplitudes greater than 50% the response, decreased. Figure 4.15 shows the degradation of the response for 5 s sonication using increasing amplitudes.



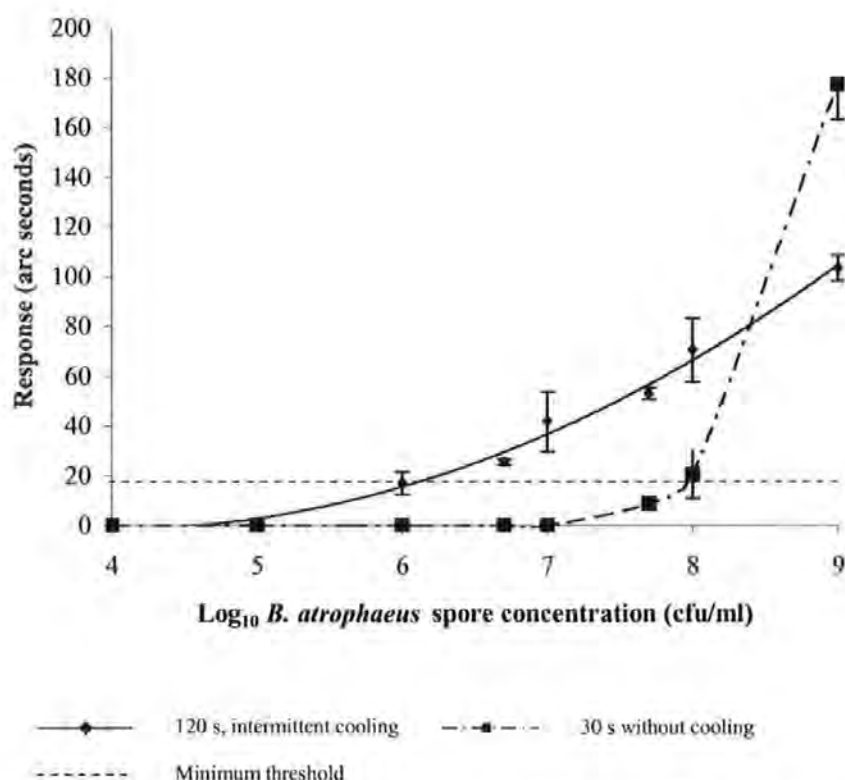
**Figure 4.15** Degradation in the response of the resonant mirror biosensor to the detection of  $1 \times 10^8$  cfu/ml *B. anthracis* spores varying the amplitude of Cepheid sonication for 5 s. The antibody used was a monoclonal anti-*B. anthracis* antibody (Dst1) which was immobilised onto a low molecular weight (T70) carboxymethylated dextran cuvette. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.3.4 Evaluation of the effect of heating and cooling

The results from both immunoassay-based techniques (ELISA and the resonant mirror biosensor) suggested that heat could be an important factor in the degradation of the response observed in these assays. Therefore a method of sonicating and cooling was employed. Samples of *B. atrophaeus* spores were sonicated in 5 s pulses at 100% amplitude and held on ice for one min between each pulse until the required time period of sonication had been achieved. The samples were then analysed by protein assay, SDS-PAGE and resonant mirror biosensor analysis. Figure 4.16a shows a higher concentration of protein was released from *B. atrophaeus* spores after 120 s sonication with pulsing and cooling (~70 µg/ml), compared to sonication without (~55 µg/ml). The amount of protein released from *B. anthracis* spores showed a similar trend; however, the maximum amount of protein released with pulsing and cooling was higher, ~200 µg/ml (Figure 4.16b). The samples demonstrating the optimal release of protein for sonication with the incorporation of a cooling step using the Cepheid system were evaluated using the resonant mirror biosensor. Figure 4.17 shows Cepheid sonication for a total of 120 s in bursts of 5 s with intermittent cooling for 1 min produced a 100-fold increase in the detection of *B. atrophaeus* spores.



**Figure 4.16 Protein release from a) *B. atrophaeus* spores and b) *B. anthracis* spores sonicated using the Cepheid mini-sonicator at 100% amplitude for varying time periods with and without cooling.** Spores (at  $1 \times 10^9$  cfu/ml) were sonicated without pulsing were sonicated continually for a defined time period. Samples sonicated with pulsing and cooling were sonicated in 5 s pulses with intermittent cooling on ice for 1 min. Protein concentrations were determined using a BCA assay. Error bars show  $\pm 2$  standard deviations from the mean.



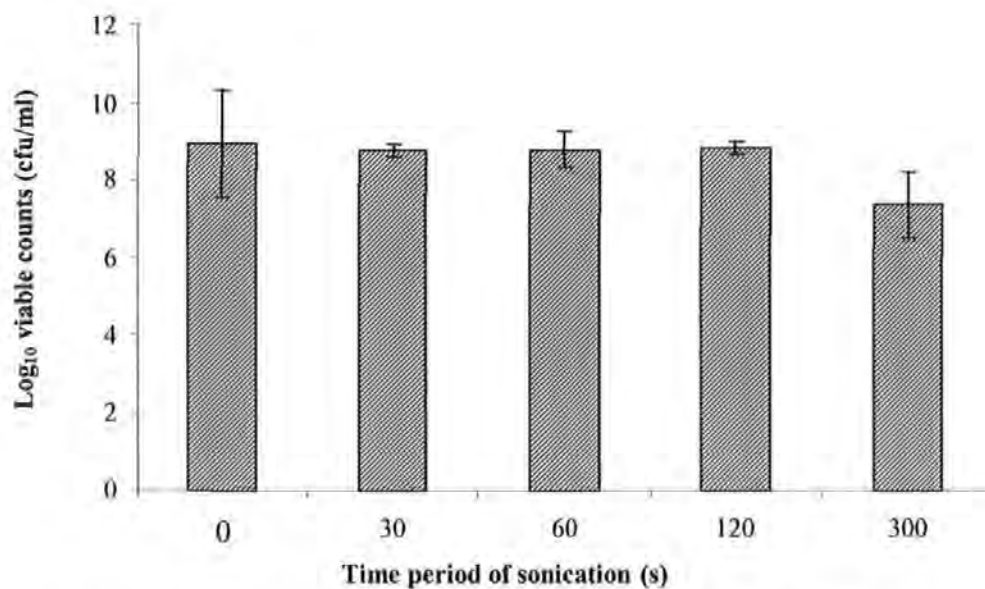
**Figure 4.17 Detection of sonicated *B. atrophaeus* spores using the resonant mirror biosensor.** Washed spores ( $1 \times 10^9$  cfu/ml) were Cepheid sonicated for 30 s at 100% amplitude or for 24 x 5 s pulses for a total of 120 s sonication, with intermittent cooling on ice for 1 min. The samples were then passed over polyclonal anti-*B. atrophaeus* antibody (Dst1) immobilised onto a T70 CMD cuvette. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.4 Evaluation of the tubular sonication system using *B. atrophaeus* spores

Although evaluation of the Cepheid sonicator showed improvements in immunoassay sensitivity, heat appeared to be a major limitation. Therefore in collaboration with Prof. T. Coakley and K. Borthwick (University of Cardiff) a novel system was developed to limit heating of the bulk solution to minimal levels. The design and development of the tubular sonicator is detailed in section 3.18. Following initial development the system was characterised using total and viable counts to determine spore breakage.

#### 4.2.4.1 Assessment of viability

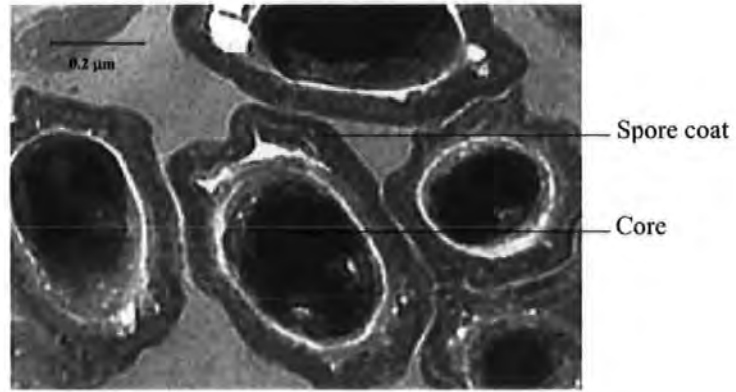
There was no statistically significant decrease in the viability of *B. atrophaeus* spores after sonication for any of the time periods tested (Figure 4.18). The statistical significance of the results was determined using the analysis of variance (ANOVA) assuming  $P < 0.05$  ( $P = 0.0785$ ). As no decrease in viability was observed total spore counts were not performed.



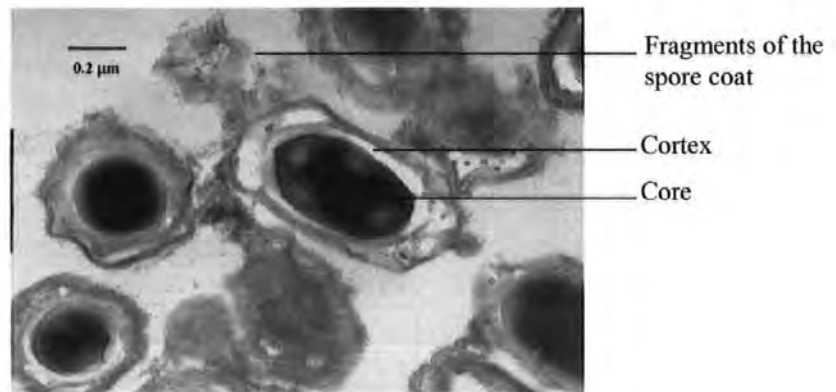
**Figure 4.18** Viable counts of *B. atrophaeus* spores after sonication for increasing time periods using the tubular sonicator. Spores (at  $1 \times 10^9$  cfu/ml) were sonicated in the tubular sonicator in batch mode, at 267 kHz, for defined time periods and the viable counts after sonication assessed. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.4.2 Electron microscopy

In the case of *B. atrophaeus* spores, observation of spore morphology after sonication with the tubular transducer for 600 s, revealed a reduction in the thickness of the spore coat. A significant amount of debris, presumably fragments of the spore coat, was also observed but there was no evidence for significant breakage of the spores (Figure 4.19b). In the case of *B. anthracis* UM23Cl2, many of the spores were devoid of an exosporial layer seen as fragments within the sections. There was also some possible damage to the spore coat. A representative section is shown in Figure 4.20.

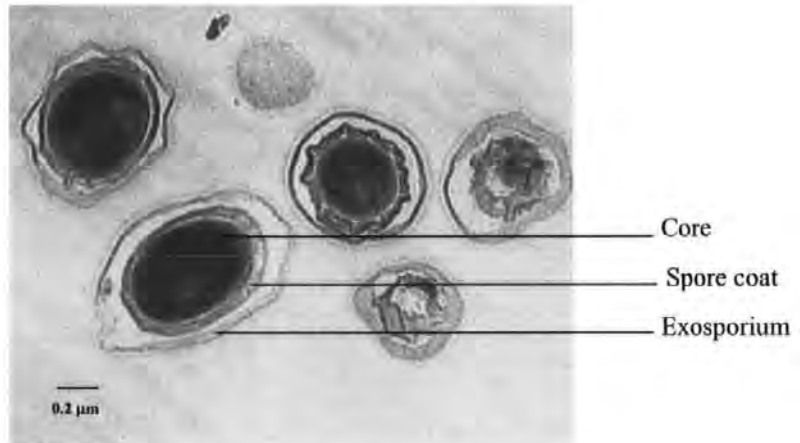


a)

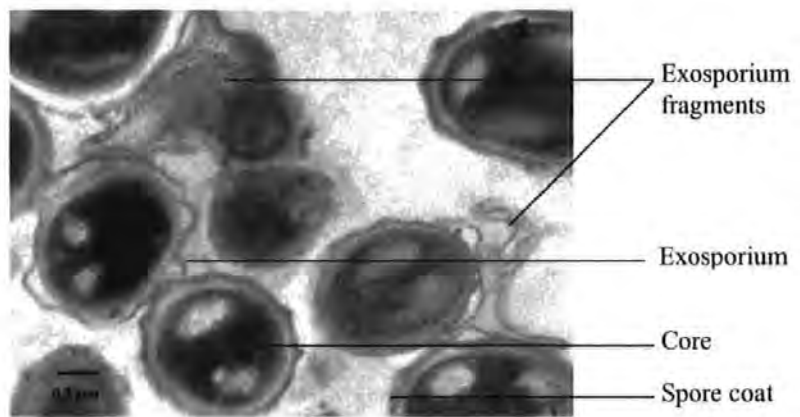


b)

**Figure 4.19** TEM analysis of *B. atrophaeus* spores before and after sonication using the tubular sonicator. a) untreated *B. atrophaeus* spores, b) *B. atrophaeus* spores sonicated for 300 s.



a)



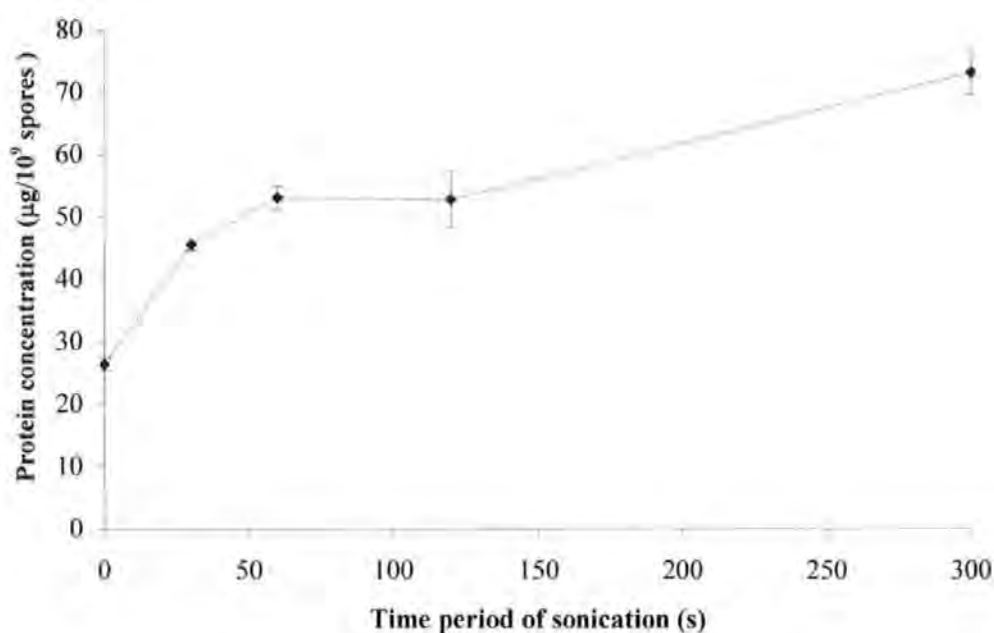
b)

**Figure 4.20** TEM analysis of *B. anthracis* UM23Cl2 spores before and after sonication using the tubular sonicator a) untreated spore b) spores sonicated for 300 s.

#### 4.2.4.3 Protein release

The amount of protein released after sonication for increasing time periods was evaluated through the use of BCA assay and SDS-PAGE. It can be seen that the amount of protein released increases with increasing time period of sonication. As observed with the standard probe and Cepheid system, there was an initial rapid increase in the amount of protein released over increasing time periods. However, it should be noted that the amount of protein released was approximately the same as that observed for the Cepheid system (Figures 4.21). This was supported by results obtained by SDS PAGE.

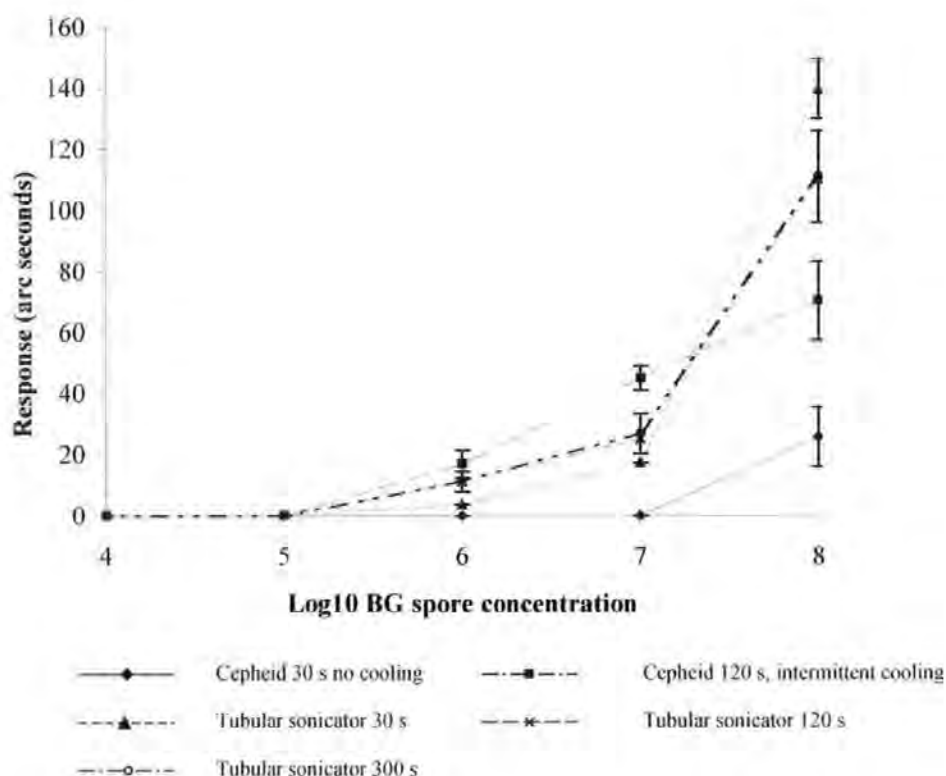




**Figure 4.21** Protein release from *B. atrophaeus* spores sonicated using the tubular sonication system for various time periods. Washed spores ( $1 \times 10^9$  cfu/ml) were sonicated in the tubular sonicator at 267 kHz for increasing time periods. Protein concentration was determined using a BCA assay. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.4.4 Enhancement of specific detection after sonication

After sonication of *B. atrophaeus* spores using the tubular sonicator the sensitivity of the resonant mirror biosensor specific immunoassay decreased slightly compared to the sensitivity obtained through the sonication of *B. atrophaeus* spores using the optimised Cepheid minisonicator protocol (i.e. with incorporation of cooling) (Figure 4.22).

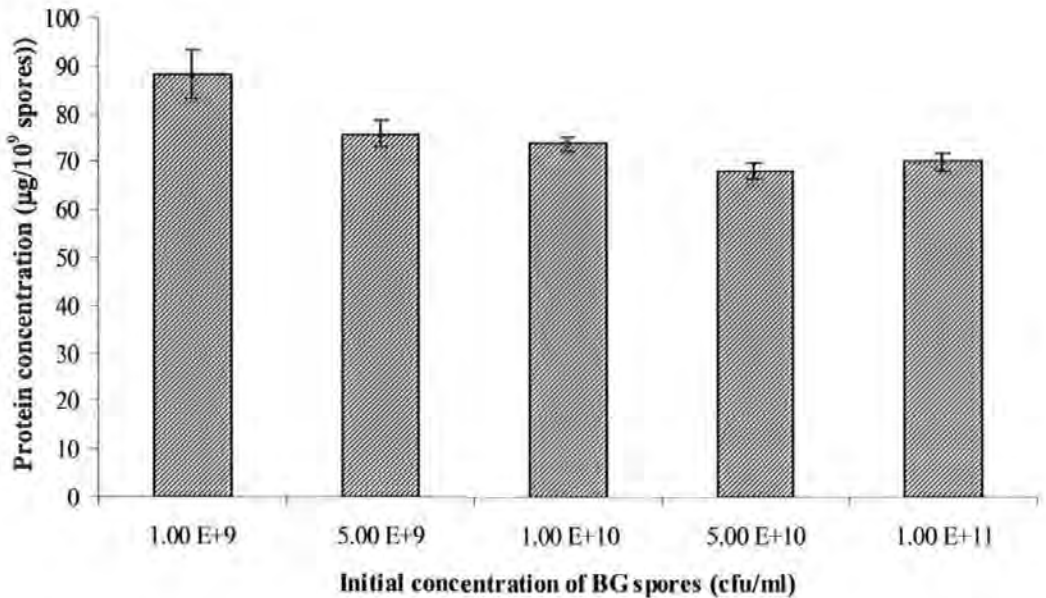


**Figure 4.22** Detection of *B. atrophaeus* spores using the resonant mirror biosensor comparing, sonication using the tubular sonicator and the Cepheid mini sonicator with and without cooling. Washed spores were sonicated at  $1 \times 10^9$  cfu/ml using; (a) the Cepheid system at 100% amplitude for 30 s without cooling (the optimised uncooled protocol), (b) with cooling for 120 s and, (c) using the tubular sonicator 267 kHz. The samples were then passed over polyclonal rabbit anti-*B. atrophaeus* antibody immobilised onto a T70 CMD cuvette. Error bars show  $\pm 2$  standard deviations from the mean.

#### 4.2.4.5 The effects of concentration on protein release

If a system is to be utilised for the detection of BW agents (in particular spores), it has to be used in a range of environments (e.g. an air sample collected into liquid, or a laboratory sample) and. Also, particularly in the case of environmental samples, other bacteria, proteins or other particulate matter may be present as part of the biological and other background. Initial experiments were conducted to examine the efficiency of the release of proteins from various concentrations of *B. atrophaeus* affected the outcome of sonication. The effects of concentration on the sonication process were assessed by monitoring protein release. There appeared to be an inverse relationship between the concentration of protein

released when various concentrations of *B. atrophaeus* spores were subjected to cavitation using the tubular sonicator. After adjustment of the protein concentrations to represent that released from  $10^9$  spores it was apparent that when the spores were sonicated at concentrations above this; the higher the initial spore count prior to sonication, the lower the concentration of protein released. The optimal concentration of *B. atrophaeus* spores was in the order of  $1 \times 10^9$  spores/ml (Figure 4.23).



**Figure 4.23** Protein release from *B. atrophaeus* spores sonicated using the tubular sonicator sonication system, sonicating for 300s, using varying concentrations of *B. atrophaeus* spores. Spores were sonicated in the tubular sonicator at 267 kHz using increasing concentrations of spores ( $1 \times 10^9$  to  $1 \times 10^{11}$  cfu/ml). Following sonication the protein concentration was adjusted to represent the protein released from  $1 \times 10^9$  spores/ml and was determined using a BCA assay. Error bars show  $\pm 2$  standard deviations from the mean.

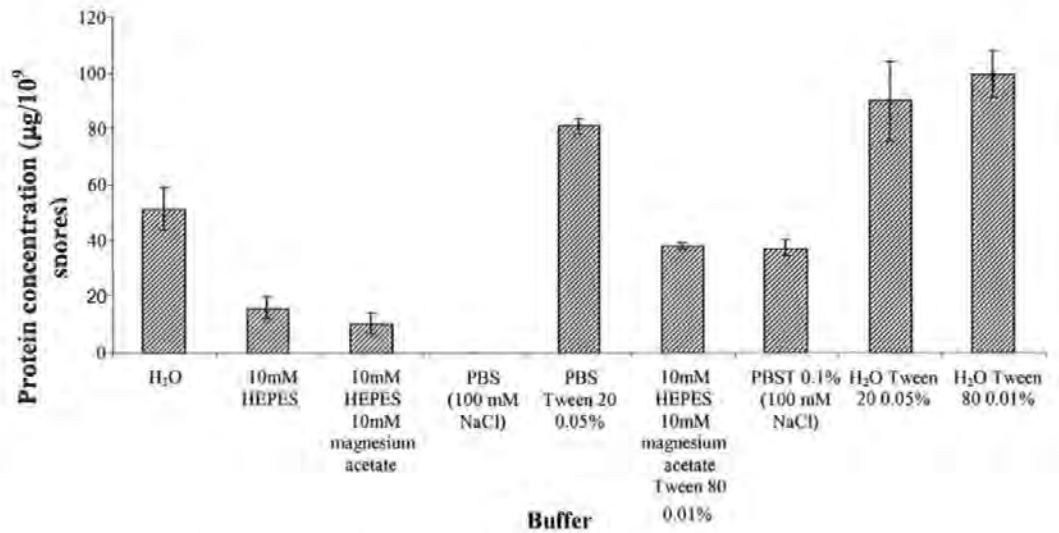
## **4.2.5 Evaluation of the tubular sonicator operated as a flow through system**

### **4.2.5.1 Evaluation of concentration of protein released**

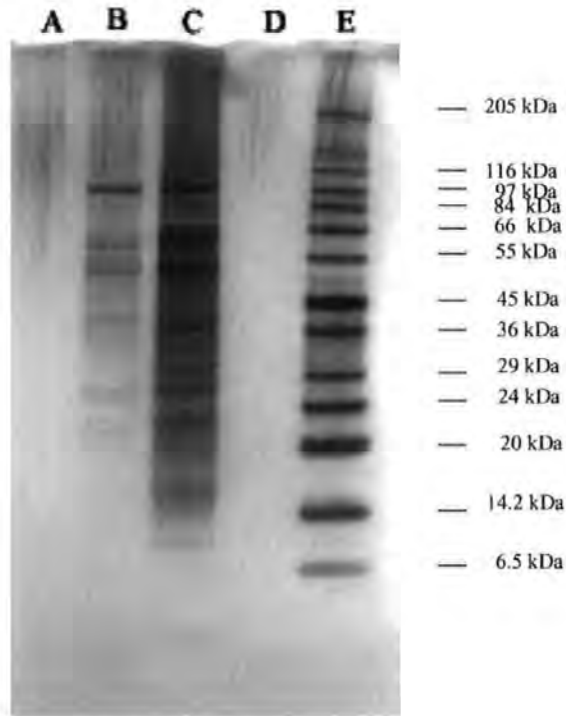
The amount of protein released from the tubular sonicator when operated in a batch mode for 300 s was approximately 75  $\mu\text{g}/10^9$  spores. However, when the sonicator was operated as a continuous flow system the amount of protein released was approximately 50  $\mu\text{g}/10^9$  spores, even though the residence time within the sonic zone was 300 s.

### **4.2.5.2 Evaluation of the use of different buffers and the addition of detergents in sonication**

A continuous or batch system may require sonication in a range of different buffers, dependent on the method of collection of the sample and the final assay format to be utilised. The effect of the addition of different buffers and detergent concentrations on the sonication of *B. atrophaeus* spores was evaluated. Buffers and detergent concentrations were selected on the basis of common usage within biosensor systems. The results show that, in the absence of detergents, the release of protein decreased as the ionic strength of the buffer increased (Figure 4.24). The addition of Tween also significantly increased the release of protein from *B. anthracis* spores (Figure 4.25).



**Figure 4.24 Protein release from  $10^9$  *B. atrophaeus* spores by sonication in different buffers and with the addition of increasing amounts of detergent using the tubular sonicator..** Washed *B. atrophaeus* spores were sonicated (at  $1 \times 10^9$  cfu/ml) in water, 10mM HEPES or PBS with the addition of various concentrations of Tween 20 using the tubular sonicator at a flow rate of 0.3ml/min at 267 kHz at 800 mV. Protein concentration was determined using a BCA assay. Error bars show  $\pm 2$  standard deviations from the mean.



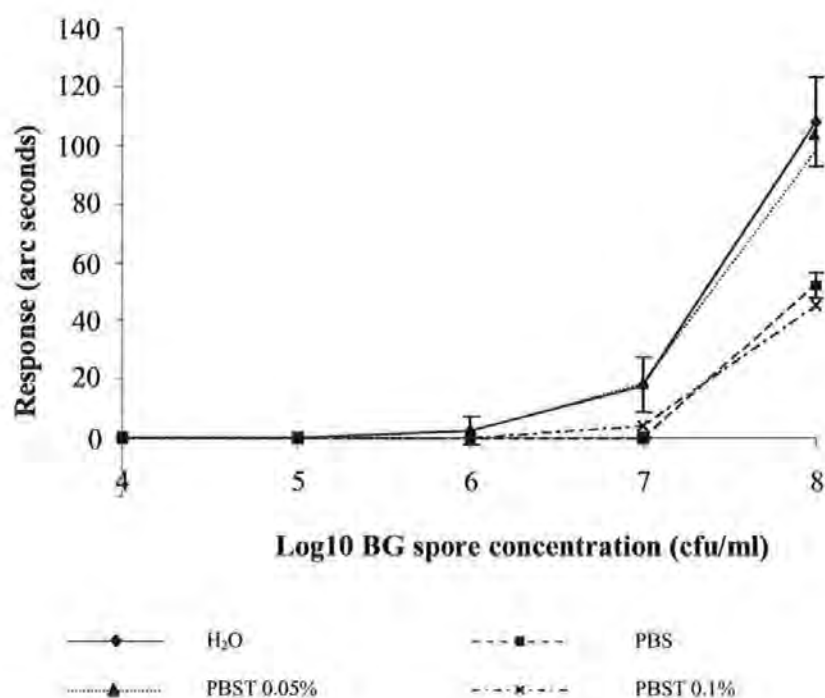
**Figure 4.25 SDS-PAGE analysis of the effects of various detergents on the release of proteins from *B. anthracis* UM23C12 spores by sonication.** Washed spores were sonicated (at  $1 \times 10^9$  cfu/ml) in water with or without Tween 20 (0.05% v/v) using the tubular sonicator at a flow rate of 0.3ml/min at 267 kHz at 800 mV. A) *B. anthracis* UM23C12 in H<sub>2</sub>O untreated control, B) *B. anthracis* UM23C12 sonicated in H<sub>2</sub>O, C) *B. anthracis* UM23C12 sonicated in H<sub>2</sub>O Tween 20 0.05%, D) reducing sample buffer. E) Wide range molecular weight marker (Sigma).

#### **4.2.5.3 The effect of the addition of protease inhibitors**

The effect of the addition of a protease inhibitor cocktail (Sigma), or 1, 2.5 5 and 10 mM EDTA before sonication was evaluated by SDS-PAGE. There was no significant difference in the protein profiles obtained with increasing concentrations of protease inhibitor compared to the untreated control (data not shown). Furthermore, there was no difference in the profile obtained if the inhibitor was added immediately after sonication.

#### **4.2.5.4 Enhanced specific detection after sonication**

From analysis using the resonant mirror biosensor, the sensitivity of the detection of *B. atrophaeus* spores showed a 20-fold reduction in assay sensitivity when the tubular sonicator was utilized in continuous flow, compared to its operation in a batch format. However, it should be noted that the sensitivity of the assay when the tubular sonicator was used as a continuous system is the same as that for 30 s sonication using the system in batch mode. This supports the results obtained from the evaluation of protein released after sonication, which again were similar for the continuous system and the batch system after 30 s sonication. Examination of the effect the addition of detergent on assay sensitivity reflected the increased amount of protein released, an example of which is shown in Figure 4.26. With *B. anthracis* the improvements in assay sensitivity were similar although the lowest detectable concentration was lower as the assay was more sensitive than that for *B. atrophaeus* spores when untreated spores were used.



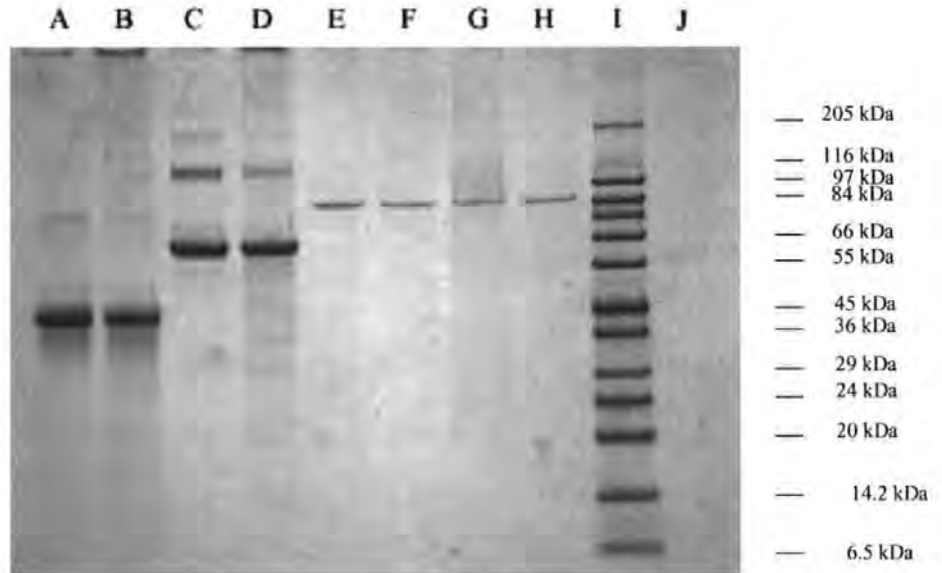
**Figure 4.26** Detection of sonicated (continuous flow tubular sonicator) *B. atrophaeus* spores using the resonant mirror biosensor in water and phosphate buffered saline (PBS) with two different concentrations of Tween 20. The spores were sonicated in the tubular sonicator in continuous flow at 267 kHz. Samples were passed over polyclonal anti-*B. atrophaeus* antibody (Dst1) immobilised onto a T70 CMD cuvette. Error bars show  $\pm 2$  standard deviations from the mean. Samples were sonicated in water (H<sub>2</sub>O) or phosphate buffered saline (PBS) or PBS with 0.05 or 0.1% (v/v) Tween 20 (PBST)

#### 4.2.5.5 Assessment of the effects of sonication on a specific protein

An assessment of the effects of ultrasonic cavitation on individual proteins was completed through the sonication of three proteins of increasing molecular weight using the tubular sonicator for increasing time periods. The three proteins chosen for evaluation were BSA (m.w. ~ 36 kDa), ovalbumin (m.w. ~ 45 kDa) and *B. anthracis* S-layer protein EA1 (m.w. ~ 92 kDa). The samples were analysed by SDS-PAGE to compare differences in the protein profiles. The S-layer protein derived from *B. anthracis* was also evaluated by resonant mirror before and after sonication using a single chain anti-EA1 antibody (Chapter 5), to evaluate antigen denaturation. Evaluation of the protein profiles obtained through SDS-



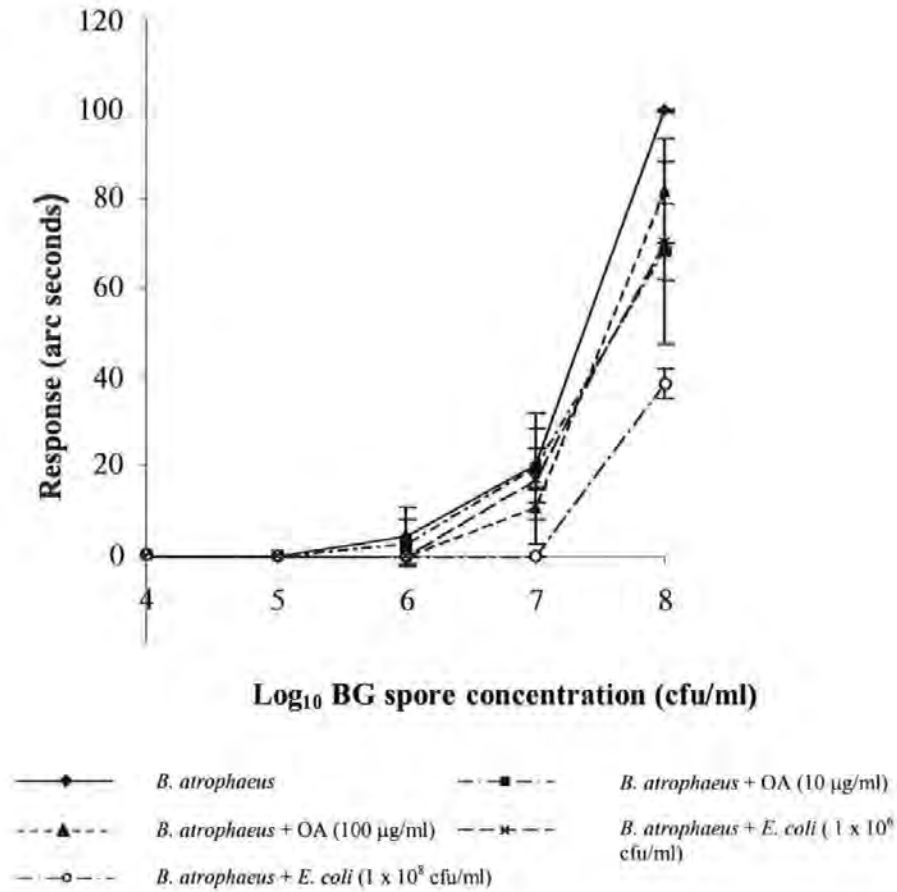
PAGE of the three sonicated proteins showed some evidence of smaller and larger fragments in the sonicated samples compared to the untreated controls (Figure 4.27). Increased 'smearing' down the gel and a greater proportion of material that was unable to enter the gel matrix was also noted in the case of BSA and ovalbumin.



**Figure 4.27** Effects of ultrasonic cavitation on proteins analysed by SDS PAGE. Samples were sonicated using the tubular sonicator in continuous flow. Following recovery of the sample (diluted 1/20 to give an expected concentration of 25  $\mu\text{g/ml}$ ) proteins were separated by SDS-PAGE on precast 4-12% Bis-Tris gels (Invitrogen) and stained with Colloidal Blue stain (Invitrogen). Lane loading was as follows: A) BSA untreated, B) BSA sonicated C) Ovalbumin untreated D) Ovalbumin sonicated E) S-layer protein untreated, F) S-layer protein sonicated in water, G) S-layer protein sonicated in PBS Tween 20 0.05%, H) S-layer protein sonicated in PBS I) Wide range molecular weight marker (SIGMA), J). Reducing sample buffer.

#### 4.2.5.6 Assessment of the effects of mixtures on specific detection after sonication

As the concentration of spores in the sample appeared to have an effect on the efficiency of cavitation, the consequence of the presence of other microorganisms and protein within the sample was assessed. *B. atrophaeus* spores were sonicated at approximately  $1 \times 10^8$  spores/ml with the addition of high ( $1 \times 10^8$  cfu/ml) and low ( $1 \times 10^6$  cfu/ml) *E. coli* and high (100  $\mu$ g/ml) and low (10  $\mu$ g/ml) concentrations of ovalbumin. Using the resonant mirror optical biosensor, the results suggest that there was no decrease in the sensitivity of the detection of *B. atrophaeus* when spores were sonicated with a low concentration of another micro-organism (*E. coli*) or protein (ovalbumin), and showed a lowest detectable concentration of  $\sim 10^7$  cfu/ml. When high concentrations of protein were added, there was a slight decrease in the sensitivity of the assay for *B. atrophaeus* spores, although this was not statistically significant. However, the addition of *E. coli* at a high concentration appeared to cause a 5 fold decrease in assay sensitivity (Figure 4.28). The final concentration of organisms within the sample was  $2 \times 10^8$  cfu/ml, furthermore a background of  $\sim 1 \times 10^8$  organism may be unrealistically high. This does not support the previous observation that no concentration effects were observed with protein release from the sonication of *B. atrophaeus* spores alone.



**Figure 4.28** Detection of sonicated *B. atrophaeus* spores using the resonant mirror biosensor. Samples were sonicated using the tubular sonicator in continuous flow at 267 kHz. The samples used were *B. atrophaeus* at  $10^9$  cfu/ml alone and mixed with ovalbumin (OA) at 100 or 10µg/ml and *Escherichia coli* (*E. coli*) at  $10^8$  or  $10^6$  cfu/ml) The samples were then passed over polyclonal anti-*B. atrophaeus* antibody immobilised onto a T70 CMD cuvette. Error bars show  $\pm 2$  standard deviations from the mean.

### 4.3 Discussion

Much of the research that has been conducted into characterisation of electroporation focuses on the use of artificial lipid bilayer (see Miteva *et al.*, 1999). However, Troiano *et al.* (1999) suggested that this is an over simplified system. In the context of biological systems, an exact explanation and model of the effects of large pulsed electric fields is extremely difficult. The reason for this is that there are many ways in which a biological system, such as a cell membrane or spore, can be directly or indirectly altered during electroporation. The distribution of proteins within a cell membrane or spore coat or underlying structures means that the electrical and mechanical properties of the cell or spore will not be homogeneous across the surface, so exact models are difficult to apply. Furthermore, changes in the external environment due to release of proteins and other components during electroporation may also alter the effects on cells or spores (Polk, 2000). It has been hypothesised if water is used as a suspension medium the electrical discharge is transferred to the water giving it bactericidal properties. It has been postulated that this is due to the formation of ions, hydrated ions and free radicals and a more systemic effect due to the formation of shock waves and UV radiation (Efremov *et al.*, 2000), although this has never been determined experimentally. In general, the most accepted causes of reduction in cell viability from applied electric fields are (a) significant damage to cell DNA; (b) severe disruption or changes to the biochemical composition of the interior of the cell rendering it incapable of survival; and (c) irreversible mechanical disruption to a biological particle or its surface structure (Efremov *et al.*, 2000).

The bacterial endospore is very different in structure to a vegetative cell, thus the effects of applied electric field on the spore are also likely to be different. Pulsed electric fields have, in the main, been reported to have no effect on bacterial endospores (Hamilton and Sale, 1967; Knorr *et al.*, 1994 Simpson *et al.*, 1995). However, results from the work presented here suggest that effects can be seen using long pulse widths (0.1 s) and field strengths of 5 kV/cm. SEM analysis of electroporated *B. atrophaeus* and *B. anthracis* spores demonstrated that, at 5 kV/cm with increasing number of pulses, a greater amount of debris and sometimes 'pustules' were observed on the spore surface. This has also been reported for sonication (Chandler *et al.*, 2001). This would suggest that material is being released into the surrounding medium and changes are occurring on the spore surface. Whether these changes extend to the underlying structures has yet to be determined. The formation

of pustules could be due to a number of reasons such as aggregation of protein into microspheres and possible precipitation or an effect on the outer integuments caused by a large efflux of ions from the highly positively charged core due to the applied electric field or a charge effect on the external protein layers may allow for re-orientation of coat or exosporium proteins and/or removal from the spore (Carstensen *et al.*, 1971). However, at present these mechanisms are purely speculative. One other feature of note was the reduction in ridging along the longitudinal axis of electroporated spores compared to untreated samples. This could be due to a number of factors: (a) disruption of the interaction of spore coat proteins required for the formation of a fold (Driks, 2003); (b) an increase in spore volume due to the increased permeabilisation of the cortex and/or core (Westphal *et al.*, 2003); and (c) induction of germination by the forced efflux of components such as DPA from the core (Santo and Doi, 1974). However, to date pulsed electric field treatment has not been shown to induce germination (Barsotti and Cheftel, 1999; Pol *et al.*, 2000).

Viable and total counts of electroporated spores demonstrated a general trend in a reduction in values with increasing numbers of pulses; however, this was only statistically significant after 99 0.1 s pulses where a 10 fold reduction was observed. However to attain a reduction in the total and viable counts no other pre-treatment was required, at variance with results from previous studies (Sale and Hamilton, 1967; Knorr *et al.*, 1994; Simpson *et al.*, 1995). The differences may be a reflection of the longer pulse widths used compared to previous experiments as there appeared to be little change in morphology observed with spores electroporated with pulses 1000 fold less in duration (0.0001 s) in agreement with previous work (Hamilton and Sale, 1967; Knorr *et al.*, 1994). The results from the work presented here suggest that the initial effects of an applied pulsed electric field are sub-lethal and are observed on the outer integuments, although gradual damage to the cortex and eventually the inner membrane can not be discounted. Although the membrane is the target of electroporation this does not appear to be the case in the case with the spore.

Evaluation of the release of DPA showed an increase with increasing number of pulses at values far greater than would be expected for the number of spores broken. However, the amount of DPA released by sonication using a standard probe sonicator was lower, even



though both disruption techniques did not show a statistically significant reduction in the viable count. This suggests that in the case of electroporation, release of DPA into the medium prior to spore breakage may occur. This could be due to a number of reasons, such as heating of the bulk solution (McClain *et al.*, 2003) or the efflux of DPA from the core due to the movement of ions upon application of an electric field. The results of the biotinylation of *B. atrophaeus* spores and subsequent electroporation suggest that with the lowest exposure surface proteins were released. However, with increasing numbers of pulses the biotinylated protein was not detectable suggesting removal of the protein to the electrodes and/or denaturation of protein.

To determine the effect of electroporation on protein and DNA, ovalbumin and purified *B. atrophaeus* DNA were subject to electroporation. Following the electroporation of protein only, the results suggested that at the amount of protein released decreased as the number of pulses increased. Washing of the cuvette following electroporation demonstrated that a proportion of the protein could be recovered. Complete recovery of the protein may not be possible if it was irreversibly denatured and not detected by any of the methods used, or if the method of recovery was not 100% efficient. Purified *B. atrophaeus* DNA was not detected following electroporation by PCR, thus it is unclear whether DNA can be released from spores using electroporation. A reduction in the optical density of proteins and DNA released from vegetative cells exposed to electric fields have been reported previously (Efremov *et al.*, 2000) and were suggested to be due to denaturation and deposition of bacterial albumens due to the increase in heavy metal concentration, this could be the reason for the effects observed within this system. Following electroporation, there was no observed detection of *B. atrophaeus* spores by immunoassay using any of the conditions tested. Thus, although this technique disrupts bacterial endospores it is unlikely that the nature of the electric field required to make this possible will release detectable antigen with the systems currently available.

Initial evaluation of a standard probe sonicator did not demonstrate any enhancement to the sensitivity of the detection of *B. atrophaeus* spores even though results indicated that protein was released, suggesting denaturation of the antibody binding site. The fact that there was no statistically significant decrease in the total or viable counts suggested that this method did not result in breakage of the spores. This supports previous observations

by Berger and Marr (1960) where breakage of bacterial spores was reported to require up to one hour of sonication using a standard probe sonicator. Therefore, a minisonicator produced by Cepheid that had been reported to break *B. atrophaeus* spores after 30 s (Belgrader *et al.*, 1999) was evaluated. However, a significant proportion of *B. atrophaeus* spores were only disrupted after 120 s of sonication. This result was supported by the analysis of samples by SEM and TEM where the effect on the outer layers of the *B. atrophaeus* spores were not obvious following shorter periods of sonication (5 and 30 s), although there was some possible sloughing of the spore coat which was rougher in appearance. Sonication for 120 s had a number of effects on the spores, which were not observed to be homogeneous throughout any sample. The spore could be completely broken open to leave only debris, the outer layers could be stripped off to reveal a smoother inner layer, or the spore could remain intact and morphologically indistinct from the untreated sample (Figure 2.15f). The reason for the difference in results could be due to the use of PCR by Belgrader *et al.* (1999) to detect DNA as a marker of spore breakage following sonication. Whilst the majority of the spore DNA is located within the core, association of DNA with the outer surface of the spore has been reported (Johns *et al.*, 1994; Kuske *et al.*, 1998). Therefore, the DNA detected may have been released from the outer integuments of the spore. If this is not accounted for then incorrect assumptions of the success of a disruption technique could be made. In this study a wide range of techniques were used to determine the extent of disruption and breakage of the spores.

TEM analysis of *B. anthracis* spores after sonication using the Cepheid system showed an observable darkening of the spore core compared to the untreated sample. This may be either due to heat activation and/or sub-lethal damage of the interior due to the sonication process. Darkened spots have been observed in the protoplasts of spores subject to heat and irradiation and have been suggested to be due to aggregation of proteins located within the core as has been observed in the case of bacterial cells (Moberly *et al.*, 1966;; Zou *et al.*, 1998; Woo *et al.*, 2000). The fact that this effect was not observed by TEM of spores sonicated using the tubular sonicator, where bulk heating effects were maintained at a minimal level, suggests that the darkening of the core is probably attributable to heat rather than sub-lethal effects of cavitation within the core.

Following preliminary ELISA results, the Cepheid minisonicator demonstrated significant improvements in the sensitivity of the detection of *B. atrophaeus*, from undetectable using untreated spores to a lowest detectable concentration of  $5 \times 10^7$  spores/ml using an optical biosensor, the resonant mirror. The optimum sonication period for *B. atrophaeus* spore detection was found to be 30 s without cooling (Figure 4.14), but no reduction in viable or total counts were observed (Figure 4.9). This would suggest that protein, recognised by the antibody, was being sloughed off the spore coat causing the initial increase in assay sensitivity. Although a greater extent of protein release was observed after longer sonication periods, this may not cause a further increase in assay sensitivity if heat or other degradative effects were denaturing the antigen released. The way in which the samples are sonicated using the Cepheid system requires the sample to be enclosed within a sealed tube. Therefore, it is likely that the build up of heat during the sonication process using the Cepheid system is greater than that observed with other methods of sonication (vonn Sonntag *et al.*, 1999). The results for *B. anthracis* showed a 20 fold increase in assay sensitivity by resonant mirror immunoassay after sonication at 50% amplitude for 5 s. Furthermore, the effect of denaturation of antigen shown by the observed degradation in the sensitivity of the resonant mirror immunoassay was observed above 50% amplitude in contrast to *B. atrophaeus* spores where the sensitivity decreased at 100% amplitude after 30 s sonication. This is probably due to the antibody used and in particular the characteristics, especially thermal stability, of the antigen recognised (Caldarelli and Los Rios, 2001).

The development of the protocol to incorporate one minute cooling phases after each 5 s pulse of sonication, demonstrated further improvements in sensitivity compared to a sonication protocol with no cooling. The observation of an apparent increase in released protein concentration with cooling suggests that heat caused the severe denaturation of proteins, possibly leading to aggregation and/or precipitation. Although a large improvement in the sensitivity of the detection of both *B. atrophaeus* and *B. anthracis* spores after sonication using the Cepheid system was observed the heat generated by the process appeared to have a limiting effect. This effect increased with increasing power amplitude and with extended time periods of sonication. However, the incorporation of cooling into the sonication protocol increased the total time period of sonication to unacceptably high levels.



To address the issue of heat denaturation of antigen, a novel design of a tubular sonicator was developed to limit temperature increases during sonication. This would permit the development of rapid assays without delays introduced by cooling requirements and allow for characterisation of the effects of cavitation excluding the effects of heating of the bulk solution. This system allowed for sensitive detection of *B. atrophaeus* spores, even though the concentration of protein released was not significantly increased, from a maximum of approximately 70 µg/ml using the Cepheid system to a maximum of approximately 80 µg/ml with the tubular sonicator. This again supports the observation that heat is one of the major degradative effects observed on proteins. An additional advantage of the tubular transducer was that the design was readily applicable to a flow through format. Using the system in continuous flow did, however, result in an approximate two-fold reduction in assay sensitivity by resonant mirror. The reason for this could be due to a slight decrease in the residence time within the sonic zone, in comparison to the previous batch system.

Because heating of the bulk solution was limited using the tubular sonicator it can be concluded that sonication only had an effect on the outer integuments of the spore, probably due to cavitation events within the suspension media. In the case of *B. atrophaeus* spores some damage to the spore coat was observed. In the case of *B. anthracis* spores complete removal of the exosporium was observed in many of the spores. However, no significant breakage of the spores was observed using the conditions tested, in agreement with the viable and total count data. Also of note was possible relaxation of the cortex in the case of *B. atrophaeus*, which has been noted before by Celandroni *et al* (2004) after exposure to heat. Although this system was devoid of bulk temperature increases, hot spots generated by cavitation are likely to form during treatment. However, the fact that this was not observed in *B. anthracis* spores where the spore coat remained largely intact, suggests this effect may be due to removal of the coat and may serve as increased protection for the spore once the coat has been removed or partial degradation of the cortex layer. Further experiments would be needed to evaluate these hypotheses. Sonication of *B. atrophaeus* spores and *B. anthracis* spores within the tubular sonicator also appeared to cause a slight reduction in the degree of ridging on the spore surface. Again, it could be speculated that this effect was due to the stresses that the spore is subject to on exposure to cavitation,

although in this case the exact nature of the stresses are difficult to define due to the multitude of possible effects of each disruption system. However, localised high temperatures, removal of spore coat proteins, in particular those important for ridge formation, and possibly other forms of damage such as oxidation may have an effect. No significant morphological differences were observed in the core of sonicated spore, compared to the untreated controls, again suggesting that cavitation events within the core are limited or do not occur, probably due to the dehydrated state of this structure.

To examine the increase in sensitivity of other immunoassay based techniques the tubular sonicator was evaluated with Threshold<sup>®</sup>, in a flow through format, with the use of *B. anthracis* spores. In this instance a ~ 100 fold improvement was demonstrated after sonication. This improved with the addition of detergent to a further 20 fold improvement in the sensitivity of the assay. In the case of the lateral flow assays a significant improvement in sensitivity (500-fold) was also observed using *B. anthracis*. Furthermore, it was noted, in the course of the experiments, that the time required for the development of the assay at high concentrations was considerably lower than that observed with the untreated control. The reason for this was probably due to the fact that the sonication procedure released proteins, therefore, wicking of antigen down the membrane to the antibody was faster than that observed with a whole spore. Thus, not only was the assay sensitivity increased but the assay time was reduced. It should be noted that in all of the immunoassay formats tested, no non-specific binding, to antibody, of components released during sonication was observed.

As the tubular sonicator did not generate excessive temperature rises, this was used to evaluate the effects of sonication on proteins as this system. From the preliminary analysis of sonicated proteins by SDS PAGE and there did appear to be some evidence for alteration of proteins during sonication. However, it appears that this is not significant enough to reduce the assay sensitivity using the resonant mirror. Furthermore, the addition of protease inhibitors and anti-oxidants did not improve assay sensitivity, suggesting either that; free radicals and proteases were not released or that they did not have a significant effect on the antigen released. Results obtained by SDS PAGE suggested that protein aggregation and possible precipitation occurred, as suggested by Strathopoulos *et al.* (2004)

as additional bands were observed accompanied by a greater proportion of material that did not enter the gel matrix. However, no differences were observed in immunoassay sensitivity for sonicated proteins. This suggests the effect of ultrasonic cavitation on the protein was negligible in the tubular sonicator using the conditions tested. Alternatively, there may have been alterations to the protein, but no denaturation of the antibody binding sites. These observations could have been due to a number of effects either singularly or in combination: (a) cleavage of higher molecular weight proteins; (b) denaturation of protein; or (c) oxidation of the proteins.

The increase of ionic strength can be advantageous for certain detection platforms such as the resonant mirror when dextran is employed, as it has been found to decrease the degree of non-specific binding and increase assay sensitivity if the protein to be detected has a low pI (Dstl, Detection Dept, unpublished data). Within this system the non-ionic detergent Tween was added to decrease the degree of non-specific interactions. However, the addition of detergent within the sample may have an advantageous or deleterious effect on the outcome of sonication, and consequently the sensitivity of subsequent assays. In general the higher the ionic strength of buffer the lower the amount of soluble protein released, resulting in lower assay sensitivity. The reason why this occurred is unclear, and requires further investigation. The addition of detergent during sonication did improve the yield of protein and the immunoassay sensitivity for *B. atrophaeus* and *B. anthracis* spores. The reason for this observed difference could be that the presence of the detergent in the liquid medium may enhance the stability of the bubbles formed during cavitation. Thus at the point of collapse the bubble is larger and hence a greater amount of energy released. Hence, the effects of microstreaming and resultant erosion of the spore surface, is greater. Alternatively there may be an increase in the proportion of bubbles acting as nuclei increasing the number of cavitation events. The addition of detergents may also stabilise proteins during sonication and limit protein aggregation. This occurs as denaturation of the protein may expose hydrophobic regions that are usually shielded within the centre of a molecule. If the hydrophobic regions of the proteins are exposed they may interact and cause aggregation of the protein, and the presence of detergents may limit this effect (Caldarelli and Los Rios, 2001).

Initial evaluation of the effects of sonication of mixtures of microorganisms by resonant mirror analysis suggests that the presence of additional bacteria can cause a slight decrease in the assay sensitivity. It has been suggested that higher concentrations of particles may protect other particles from any degradative effects of sonication; however, this would appear not to be the case. The results here suggest that when higher concentrations of bacterial spores were sonicated the release of protein by ultrasonic cavitation was less efficient, and the assay sensitivity decreased. This is most likely due to the increased viscosity of the sample reducing cavitation events (Khistmatullin, 2004).

*B. atrophaeus* was chosen as a model organism to evaluate the range of disruption techniques. Furthermore, as this organism is used to evaluate a wide range of detection technologies within the field, characterisation of the effects of disruption was required for future work. The most significant effects on the differences between *B. anthracis* were observed within the Cepheid system. In this system the amount of protein released was far lower than that observed with *B. atrophaeus*, furthermore the degradation in the resonant mirror immunoassay sensitivity was at lower amplitudes of sonication and lower time periods. However, in the tubular sonication no degradation in assay sensitivity was observed at the increasing time periods tested for *B. atrophaeus*. This suggests that the consequences of sonication, (such as heat, shown to have the most significant effect) had a greater influence on the sensitivity of an immunoassay on *B. anthracis* than *B. atrophaeus*. However, in the case of the tubular system, the results appear comparable. This does however highlight the need for caution in the choice of a 'model organism' when characterising systems. The choice of antigen –antibody pair will also have an effect dependent on the stability of the antigen to the stresses it is subject to during cavitation. This highlights the need for careful selection and characterisation of proteins for sensitive detection. Proteins released during the disruption process could be identified and characterised for high resistance to ultrasonic denaturation so that appropriate recognition elements could be produced. Identification of proteins that were released at high concentrations and production of suitable recognition elements against these proteins could enhance assay sensitivity further.

In conclusion, although electroporation was shown to release surface located proteins, these proteins were subsequently undetectable by immunoassay due to significant

denaturation and/or deposition on the electrodes. For the use of electric fields to be fully explored a range of different pulse shapes lengths and field strengths would have to be evaluated and may be significantly different for any given organism or protein. The results of this work clearly demonstrate that sonication allowed for the sensitive and rapid detection of *B. atrophaeus* and *B. anthracis* spores, and this was selected as the method of choice for further investigations.

## Chapter 5 Identification of proteins of the *Bacillus anthracis* spore

### 5.1 Introduction

In *B. anthracis* and other members of the *B. cereus* group, the surface of the outer spore coat is covered by a loose balloon like structure, termed the exosporium (Ohye and Murrell, 1973; DesRosier and Lara, 1984; Driks, 1999), which surrounds, but is not attached to, the other spore components (Gerhardt, 1967; DesRosier and Lara, 1984). The exosporium has a close-packed hexagonal lattice structure consisting of a paracrystalline basal layer and an outer nap of fine filaments (Roth and Williams, 1963; Gerhard and Ribi, 1964) and is composed of protein, lipid and carbohydrate (Matz *et al.*, 1970; Beaman *et al.*, 1971).

Since the exosporium forms the outermost integument between the spore and its external environment, antigens located within the exosporium could be useful as targets for detection and diagnostic purposes (Steichen *et al.*, 2003). Some studies have suggested that the exosporium and components that may not be structural elements but associated with this outer layer, may be important for a number of functions within the macrophage due to the apparent tight interaction of the exosporium with the phagolysosomal membrane that has been observed using transmission electron microscopy (Dixon *et al.*, 1999; Guidi-Rontani *et al.*, 2001). Therefore, identification of exosporial antigens may also be important for the understanding of *B. anthracis* pathogenesis and the development of improved therapeutics in the treatment or prevention of anthrax infection.

The first structural exosporium protein to be identified in *B. anthracis* was BclA, a collagen-like glycoprotein consisting of a number of centrally located GXX (usually GPT) repeats (Sylvestre *et al.*, 2002). This protein was found to be the primary structural component of the filaments of the hairy nap. The exact function of BclA has yet to be elucidated. Sylvestre *et al.* (2002) demonstrated that, although the protein has a clear structural role within the exosporium, it is not essential for virulence in a subcutaneous model of infection in mice and does not contribute to the resistance of spores to harsh chemical treatment. BclA displays considerable size heterogeneity among the different *Bacillus* species and strains; the protein has different numbers of GPT repeats and [GPT]<sub>5</sub>GDTGTT repeats, and it has been hypothesised that these variations could play a role in the properties of the spores in different environments (Sylvestre *et al.*, 2003). Steichen *et al.* (2003) demonstrated that BclA was the immunodominant protein within the

exosporium of *B. anthracis*. As such, it was suggested that this protein may represent a potential marker for immunoassay based detection and diagnosis of anthrax infection. However, the protein is not unique to *B. anthracis*, since it has also been shown to be present in *B. cereus* and *B. thuringiensis* (Castanha *et al.*, 2006; Redmond *et al.*, unpublished data).

A number of studies have been conducted to identify further proteins of the *B. anthracis* exosporium and spore coat utilising a range of techniques. These have predominantly involved extraction of the spore coat or exosporium fraction, separation of the proteins by one-dimensional (1D) gel electrophoresis and identification of the proteins by N-terminal sequencing or mass-spectrometry (Lai *et al.*, 2003; Steichen *et al.*, 2003; Todd *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004). Many of the proteins identified are as yet uncharacterised, and their immunogenicity and suitability as markers for detection and diagnostic purposes has not been established. Immunogenic proteins are ideal as markers for such assays as they will produce an immune response allowing reagents such as antibodies to be developed. The production of an immune response to such markers may also suggest a role in the infection process. To date, only BclA and the ExsFA protein have been identified as both structural and immunogenic proteins of the *B. anthracis* exosporium (Sylvestre *et al.*, 2002, 2005; Steichen *et al.*, 2003). More recently, work has indicated that the ExsF proteins are required for the proper localisation of BclA to the spore surface and for the stability of the paracrystalline basal layers of the exosporium (Sylvestre *et al.*, 2005).

Although the exosporium is the outer integument of the *B. anthracis* spore its role, particularly in *B. anthracis* pathogenesis is still relatively unclear. Furthermore, many of the components of the exosporium identified to date have homologues (sharing up to 100% identity), particularly within the *B. cereus* group. Therefore, extension of the range of possible targets for detection and diagnostic purpose to moieties located in underlying structures such as the spore coat may yield detection targets that are more likely to meet the desired criteria of essential function, and being unique (or having unique regions). The aim of the work described here was to firstly examine surface labelling of *B. anthracis* spores and subsequently identify potential detection targets located on the outside of the spore, the exosporium. Secondly, an immunopurification strategy was developed using a polyclonal antibody raised against exosporium of wild type Ames strain and a simple liquid phase based separation method to identify other immunogenic proteins of the *B.*

*anthracis* exosporium. Finally, the use of sonication, using a novel radial transducer, which may extend the range of proteins released from the underlying layers, thus increasing the number of targets that could be utilised for recognition element production and subsequent incorporation into a detection assay. The implementation of computer modelling to the proteins released would allow for the identification of unique regions of a given protein to allow for the production of specific recognition elements.

## 5.2 Results

### 5.2.1 Identification of surface located proteins

Initial experiments for surface labelling were carried out using *B. atrophaeus* spores as a model system and further investigations conducted using the *B. anthracis* strain UM23C12 (pXO1/pXO2), the wildtype Ames strain exosporium and  $\gamma$ -irradiated spores. Other closely related *Bacillus* species were also used for comparison. In this work all spores were sonicated using the novel radial transducer as described in Chapter 3 (section 3.18.2).

#### 5.2.1.1 FITC labelling of spores

Analysis of the FITC labelled spores showed that the maximum amount of FITC bound to *B. atrophaeus* spores was achieved using 200  $\mu\text{g/ml}$  of FITC (analysing the absorbance of sample at 501 nm). This also produced no background within the sample as observed by fluorescence microscopy (data not shown). Following sonication of the spores the proteins released were separated from the spores and concentrated. These proteins were then separated by SDS-PAGE on a 4-12% Bis-Tris gel (Invitrogen) and the fluorescently labelled proteins visualised. A fluorescently labelled polyclonal anti-*B. atrophaeus* antibody was used as the control. No bands were observed in any of the sonicated spore samples (Figure 5.1). However, the presence of a band in the antibody control lane suggests that the FITC was at a high enough concentration for visualisation using this method.



A B C D E F G H I J

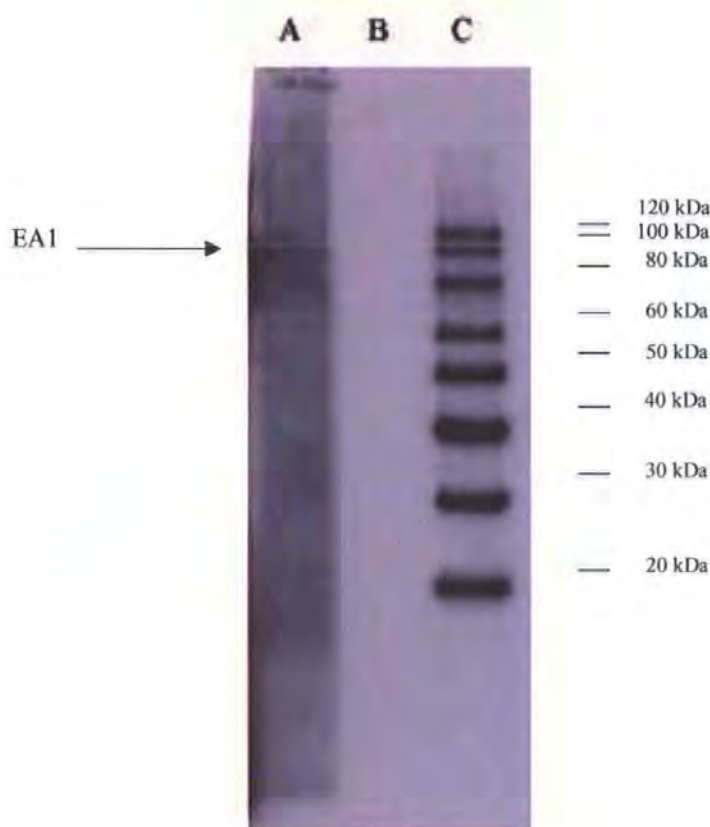


**Figure 5.1** Visualisation of FITC-labelled proteins by SDS-PAGE. Samples were separated by SDS-PAGE on a 4-12% Bis-Tris gel (Invitrogen). A) Reducing sample buffer, B) Reducing sample buffer, C) *B. atrophaeus* supernatant unconcentrated, D) *B. atrophaeus* supernatant concentrated x10, E) *B. atrophaeus* supernatant concentrated x20, F) *B. atrophaeus* supernatant concentrated x50, G) No FITC label control H) FITC-labelled antibody, I) Reducing sample buffer, J) Reducing sample buffer.

#### 5.2.1.2 Biotinylation of spores

The optimum time period for biotinylation of *B. atrophaeus* spores was 16 h. No proteins were observed in the absence of biotinylation (data not shown), suggesting that the streptavidin conjugate was not binding non-specifically to any of the proteins released.

The biotinylation of *B. anthracis* UM23C12 spore proteins and subsequent visualisation using SDS-PAGE and Western blotting techniques showed that a range of proteins were released (Figure 5.2). The predominant biotinylated proteins within the UM23C12 samples appeared to migrate at approximately 97 kDa. A fainter band was also observed at approximately 85 kDa. The other bands were significantly fainter, with 'smearing' down the gel thought to be indicative of either a significant proportion of glycosylated material within the surface proteins, an artefact of the sonication process, variability in the degree of biotinylation of each protein, or degradation of the proteins.



**Figure 5.2 Detection of biotinylated spore proteins from *B. anthracis* UM23C12 spores.** Samples were separated by SDS-PAGE on 4-12% Bis-Tris gels and blotted onto PVDF membranes. The amount of protein loaded in each lane was approximately 5  $\mu$ g. Lane loading was as follows A) Biotinylated *B. anthracis* UM23C12, B) Unsonicated, biotinylated *B. anthracis* UM23C12 spores, C) MagicMark™ Western Standard (Invitrogen).

Nano-LC mass spectrometry of in-gel digested samples failed to identify the proteins with the exception of Extracellular antigen 1 (EA1). The only other protein identification was a biotin-avidin complex.

### 5.2.2 Identification of immunogenic proteins

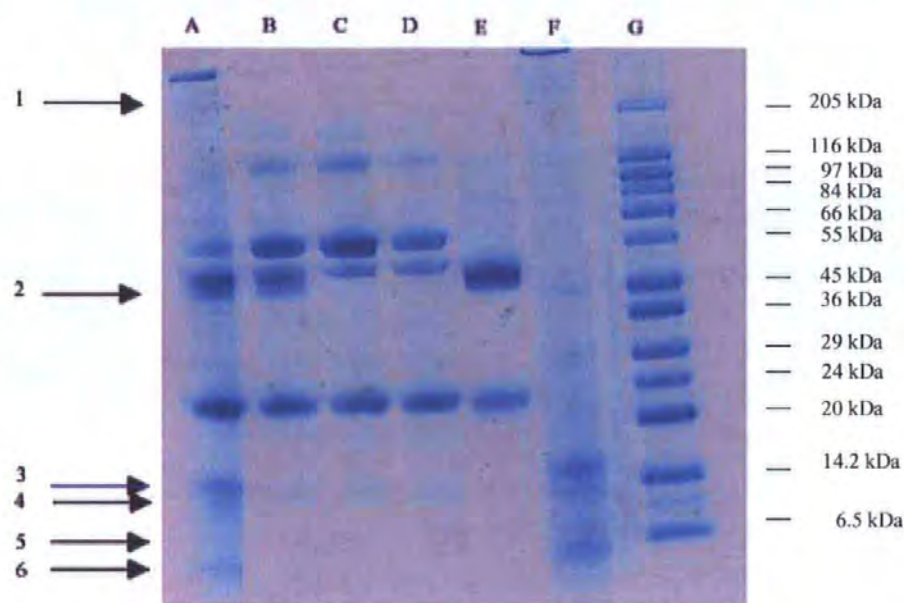
This method made use of available antibodies raised against *B. anthracis* spores. A range of antibody immobilisation strategies was investigated. Non-directed covalent coupling of antibody to a column did not yield sufficient protein for identification, probably due to the inefficiency of antibody immobilisation to the column, so beads were used to increase the available area for binding. These also provided the added advantage of the introduction of a concentration step by elution into a reduced volume compared to the original sample

volume. Since this method still yielded insufficient concentration of protein, a directed attachment approach was adopted. This involved the use of anti-species magnetic beads and an anti-exosporium antibody. Exosporium proteins which bound to the beads coated with polyclonal rabbit anti-exosporium antibody were eluted and separated by SDS-PAGE (Figure 5.3, lane A). The bands at approximately 22 and 45 kDa appeared to correspond to the anti-exosporium antibody eluting from the beads, as can be seen from the anti-exosporium antibody only control (Lane E, Figure 5.3). Four bands at 110, 55, 50 and 20 kDa and were present in the bead only control sample (Lane D, Figure 5.3). This may have been due to the polyclonal anti-species antibody eluting from the beads. A number of the eluted proteins, that were not present in the control samples, were selected from Lane A (Figure 5.3) and were identified by nano-LC-mass spectrometry and are listed in Table 3.2. No bands corresponding to eluted exosporium proteins were observed in the control samples, only the presence of antibody (Figure 5.3, lanes B and E). The incorporation of BSA and non-ionic detergent into the wash and incubation steps also appeared important in the elimination of non-specific binding of protein to the DYNAL beads (data not shown).

In order to ensure that the individual proteins that were identified were immunogenic and not simply co-eluted with the immunodominant glycoprotein BclA, Western blot analysis of the original antigen and the exosporium proteins, which bound to the anti-exosporium antibody, was conducted using the polyclonal anti-exosporium antibody used to isolate (immunopurify) the proteins. Firstly, the polyclonal anti-exosporium antibody was biotinylated and this was used to probe a Western blot onto which the proteins eluted from the DYNAL beads were transferred, following separation by SDS-PAGE. To probe the proteins a biotinylated anti-exosporium antibody was used as the primary antibody and a streptavidin peroxidase conjugate was used as the secondary detection reagent. This would reveal proteins recognised by the antibody, but would not show detect any antibody eluted during elution from the DYNAL beads. A number of proteins were observed, in particular a high molecular weight protein of approximately 250 kDa which may correspond to BclA (Figure 5.4a.). To determine if any additional proteins were present a polyclonal rabbit anti-exosporium antibody was used to probe the blot and an anti-rabbit HRP conjugate used as the secondary antibody. Again, a number of proteins were observed, and in contrast to the negative controls a number of lower molecular weight proteins were also shown (Figure 5.4b). These proteins can also be observed as faint bands in the exosporium positive sample on the gel (lane A, Figure 5.3. This was of relevance as proteins such as ExsF, ExsY and CotY have been shown to associate with BclA to form large molecular



weight complexes (Redmond *et al.*, 2004; Sylvestre *et al.*, 2005). The results shown in Figure 5.4 confirmed the immunogenic nature of the proteins identified in each band.

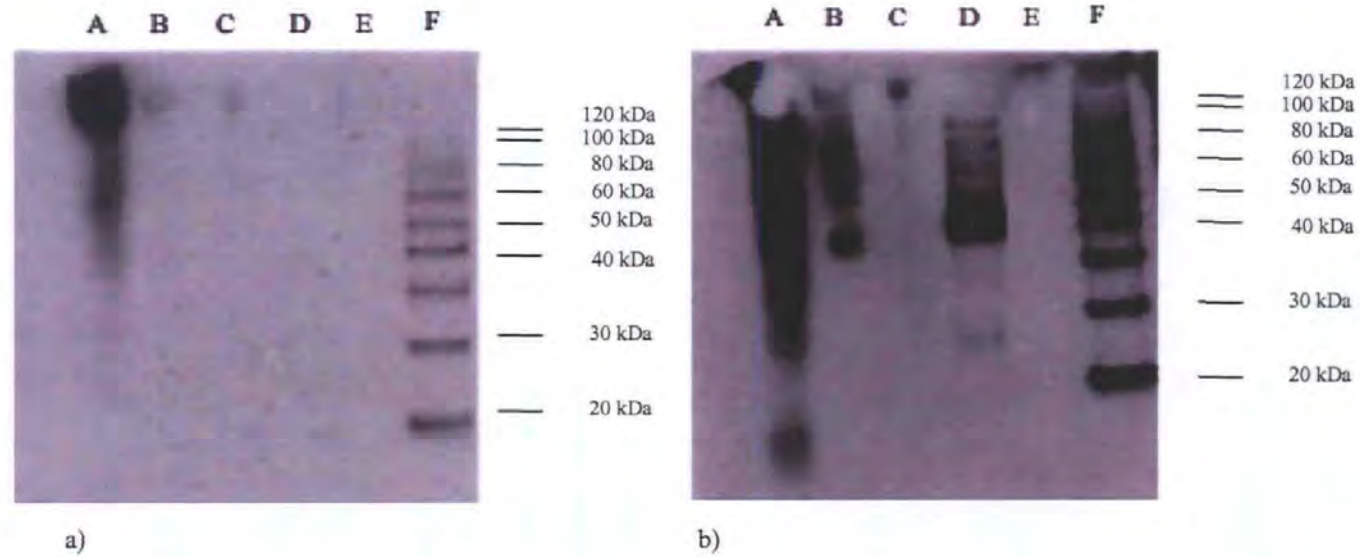


**Figure 5.3** The elution of exosporium proteins from DYNAL anti-rabbit beads with polyclonal rabbit anti-Ames exosporium antibody bound to the anti-rabbit antibody. 20  $\mu$ l of each eluted protein, 1  $\mu$ g of antibody and 5  $\mu$ g of unwashed wild type Ames exosporium were separated by SDS-PAGE on a 4-12% Bis-Tris gel (Invitrogen). Lane loading was as follows: (A) Anti-exosporium antibody +ve, exosporium +ve; (B) Anti-exosporium antibody +ve, exosporium -ve; (C & D) Anti-exosporium antibody -ve, exosporium +ve; (E) Anti-exosporium antibody only; (F) Unwashed wild type Ames exosporium only; (G) Sigma wide range molecular weight marker. The gel was stained with Colloidal Blue Stain (Invitrogen). The bands in lane A, indicated by arrows were analysed by nano-LC mass spectrometry,

Protein identification	Apparent Molecular Mass (kDa)	Predicted Molecular Mass (kDa)	Predicted pI	Peptide sequences	Protein sequence	Previously identified
BA2554 Hypothetical protein ExsK	5, 9, 14, 15, 38, 250	12	8.16	ITTGSNNYAGTVSSVTCDVVK	Band 1, 2, 3, 4, 5 and 6 MGRYSNSRKKCSCKQCCKQDDCWDVFECKKEHEEQNKACDCCCQGIRDELRLKLVNRSVRIT TGSNNYAGTVSSVTCDVVKLANASAGVTVIISVCKIEAIEALLT	Liu <i>et al.</i> , 2004; Redmond <i>et al.</i> , 2004
BA1234 ExsY	5, 9, 14, 15, 38, 250	16.1	4.99	AGAPFEAFAPSANLTSCR VESVDDDDSCAVLR	Band 1, 2, 3, 4, 5, and 6 MSCNENKHHGSSHCVDVVKFINELQDCSTTTGSGCEIIFFLGAHNTASVANTRPFILYTKAGAPFE AFAPSANLTSCRSPIFRVESVDDDDSCAVLRVLSVVLGDSPPVPTDDPICTFLAVPNARLVSTSTCITY DLSCFCAIQCLRDTI	Liu <i>et al.</i> , 2004; Redmond <i>et al.</i> , 2004
BA1238 CotY	38, 15, 14, 9, 5	16.8	4.73	AGAPFEAFAPSANLTSCR VESIDDDDDSCAVLR	Band 2, 3, 4, 5, 6, MSCNENEDHHHDCDFNCVSNVRFIHELQECATTTGSGCEVFPFLGAHNSASVANTRPFILYTKA GAPFEAFAPSANLTSCRSPIFRVESIDDDDDCAVLRVLSVVLGDTSPVPTDDPICTFLAVPNARLISTN TCLTVDLSCFCAIQCLRDTI	Liu <i>et al.</i> , 2004; Redmond <i>et al.</i> , 2004
BA0252 Alanine racemase	38	43.6	5.68	AGITAPILVLGSPPPR FQGITFNAVR GDGISYNVTYR LQGFVNLVNGK	Band 2 MEEAPFYRDTWVEVDLDAIYNNVTHIKEFIPSDVEIFA VVKGNAYGHDYVPVAKIALEAGATRLAV AFLDEALVLRRAGITAPILVLGSPPPRDINVAEENDVALTVFQKEWVDEAIKLDGSSSTKYHINFDS GMGRIGIRERKELKGFLLKSLEGAPFLELEGVYTHFATADEVTSYFDKQYNTFLEQLSWLKEFGVDP KRVHTANSAATLREQITFNAVRIGIAMYGLSPSVEIRPFLPFKLEPALSHTKVAHIKQVIKGDGISY NVTYRTRKTEEWIATVAIGYADGWLRRLOGFEVLVNGKRVPIVGRVMTDQFMHLPCEVPLGTVTL IGRQGDDEYISATEVAEYSGTINYEIITISFRVPRIFRNGKVVVEVINYLNDI	Popham <i>et al.</i> , 1996; Liu <i>et al.</i> , 2004; Redmond <i>et al.</i> , 2004; Steichen <i>et al.</i> , 2003; Todd <i>et al.</i> , 2003
BA0355 CotB homologue CotB1	15, 14, 5	19.4	5.4	DLIGSFVR GTIISVCPDYFVLQNEK GELYYYQLSHLK	Band 3, 4 and 6 MSLFHCDFLKDLIGSEVRVNRGGPESQRGTIISVCPDYFVLQNEKGELEYYYQLSHLKSITKSAKECGS SDCEWEDCACAEDFEALLESFKYCWWKINRGGPEKVEGILQDVSCDFVTLIVKEEIIIAIKHIKSVNY NALACGESDESDDSSSKESSNNSGRARAQRQSSRGR	Lai <i>et al.</i> , 2003; Redmond <i>et al.</i> , 2004
Second CotB homologue <sup>1</sup> CotB2	15	16.8	6.6	VGELVSLGK DYLTQLPHGELVYYQLK	EXXXXXLENVLCCDQIKCLVGETVKVNLRGPESRVGEVLSLGDYLTQLPHGELVYYQLKHVKS LVKKVKESKCGDCYSSCFCSDEDTFDLILKDLKYKWWKINRGGPECMDGLLSEVHHGCITLVNDCDE VIYVINSHIKSVSQVVKCKKNEDE	Not identified previously
BA2332 Hypothetical protein ExsM	15 and 14	14.4	5.36	NEVPPVETDAR	Band 3 and 4 MFFAKLRGRNEVPPVETDARGEAFFKLSRDELSLKFKLDFDIEDVVA AHLHIGAKGTNGPVVAF LF GPITNPVSIECATLTGMTQEDLVGLAGQTLGTLNEIISGNIYINVHTVQHPNGEIRGQLNYC	Redmond <i>et al.</i> , 2004
BA1237 BxpB/ ExsF/ ExsFA	15 and 14	17.3	4.27	FFLSLGTPANIIPGSGTAVR	Band 3 and 4 MFSSDCEFTKIDCEAKPASTLPAFGFAFNASAPQFASLFTPLLLPSVSPNPNITVVPVINDTVSVGDGIR ILRAGIYQISYTLTISLDNSPVAPEAGRFFLSLGTANIIPGSGTAVRSNVIGTGEVDVSSGVILINLNP DLIRIVVELIGTVDIRAAALTVAQIS	Liu <i>et al.</i> , 2004; Redmond <i>et al.</i> , 2004; Steichen <i>et al.</i> , 2003; Sylvestre <i>et al.</i> , 2005; Todd <i>et al.</i> , 2003
BA4266 Hypothetical protein Cot alpha	15 and 14	13.4	6.97	NTFTGILR	Band 3 and 4 MFGSFGCCDNFRDCHHHERERDHREREKEREVKKPQPAVCNVLASISVGTLSLLSVKGVGSFNNVIFE GFCNGVALFSALARNNDKDNKDNKDDKHNQNRNFTGILRVCPDITVAIAI	Kim <i>et al.</i> , 2004; Liu <i>et al.</i> , 2004

**Table 5.1. Immunogenic proteins identified from unwashed *B. anthracis* wild type Ames exosporium using a polyclonal rabbit anti-exosporium antibody<sup>12</sup>** The peptide sequences were used to identify the *B. anthracis* protein from the *B. anthracis* Ames genome at NCBI (NC\_003997).

<sup>1</sup> The bands of the gel refer to the following molecular weights; Band 1 – 250 kDa, Band 2 – 38 kDa, Band 3 – 15 kDa, Band 4 – 14 kDa, Band 5 – 9 kDa, Band 6 – 5kDa. <sup>2</sup> The second CotB homologue is degenerate in *B. anthracis*



**Figure 5.4 Confirmation of the immunogenic nature of bound proteins.**

The detection of bound antibody was conducted using; a) biotinylated polyclonal rabbit anti-exosporium detected by streptavidin HRP conjugate and b) polyclonal rabbit anti-exosporium detected by goat anti-rabbit HRP conjugate. 10  $\mu$ l of each eluate was run per lane by SDS-PAGE on a 4-12% Bis-Tris NuPAGE gels (Invitrogen) and blotted onto a nitrocellulose membrane (Invitrogen). Lane loading was as follows: (A) Anti-exosporium antibody +ve, exosporium +ve; (B) Anti-exosporium antibody Ab +ve, exosporium -ve; (C) Anti-exosporium antibody -ve, exosporium +ve; (D) Anti-exosporium antibody only; (E) Sample buffer; (F) MagicMark™ western standard.

### **5.2.2.1 Immunogenic proteins of the *B. anthracis* exosporium**

The band at 250 kDa (Fig 5.3 Band 1), contained sequences that correspond to previously described exosporium proteins; ExsK and ExsY (Redmond *et al.*, 2004; Liu *et al.*, 2004). Sequences derived from these proteins and an additional protein CotY were also identified in bands at 40, 15, 14, 9 and 5 kDa (Figure 5.3, bands 2, 3, 4, 5, 6 respectively).

Peptides from ExsK, ExsY and CotY were detected in the band at approximately 40 kDa (Figure 5.3, band 2) may suggest the formation of another multimeric complex, which like the complex formed between BclA and ExsY and CotY, may be resistant to denaturing SDS-PAGE (Redmond *et al.*, 2004). Alanine racemase was also identified in Band 2 (Figure 3.5) having an apparent molecular weight of 40 kDa, close to its predicted molecular weight (43.6). However, results here may indicate some form of interaction of alanine racemase with ExsK, ExsY and CotY. BLAST searches revealed ExsY and CotY have homologues with 100% and 97% identity to proteins in *B. cereus* E33L and *B. thuringiensis* serovar *konkukian* str. 97-27 respectively. ExsK has homologues that have up to 98% identity within *B. cereus* E33L, Alanine racemase has up to 99% identity with other members of the *B. cereus* group

ExsF was identified in bands 3 and 4 giving an apparent molecular weight of 14 and 15, compared to the predicted molecular weight of 17.3 kDa, this disparity could be a result of partial degradation, processing or more realistically a discrepancy due to the use of a gel electrophoresis to determine the molecular weight of the proteins and has been observed previously (Redmond *et al.*, 2004). As with alanine racemase, protein homologues sharing 99% identity, are present in the genomes of closely related *Bacillus* species.

Peptides derived from a CotB homologue (BA0355), were identified in bands 3, 4 and 6. Furthermore the presence of the peptides VGELVSLGK and DYLTQLPHGELVYYQLK in a band at 15 kDa appear to be derived from a second CotB homologue. Using the protein sequences suggested for CotB1 and CotB2 it was found that protein homologues are present in other members of the *B. cereus* group sharing up to 97% identity with both proteins.

Bands 3 and 4 (15 and 14 kDa respectively) contained the peptide sequence NEVPPVETDAR that correspond to *B. anthracis* Ames protein BA2332. These bands also contained the sequence NTFTGILR derived from BA4266, a Cot alpha homologue (Kim *et al.*, 2004). The genomes of closely related *Bacillus* species possess proteins with up to 100% identity to BA4266.

### **5.2.3 Identification of other proteins of the *B. anthracis* spore**

#### **5.2.3.1 Direct pepsin digestion and identification of proteins of the *B. anthracis* spore**

Following sonication, and pepsin digestion and analysis of the resultant supernatant, a range of proteins were identified from the *B. anthracis* spores (Table 5.1). The majority of the proteins identified have been described previously from experimental studies of the *B. anthracis* spore with the exception of the *B. cereus* ExsA homologue. BLAST searches of sequences revealed that the majority of protein demonstrated high percentages of identity (up to 100%) with homologous proteins within the *B. cereus* group. Therefore, these proteins would be unsuitable for examination as potential targets for a specific detection assay. However, three proteins BxpA and ExsA and EA1 were of interest due to the lower degree of identity shared, 91%, and 93% respectively with homologous proteins that could be exploited for the production of specific recognition elements.

#### **5.2.3.2 In-gel digestion of samples**

The sonication of spores demonstrated the release of a wide range of proteins from *Bacillus* species spores. Using SDS PAGE, alanine racemase and EA1 were the first markers to be identified on the basis of the molecular weight and this was confirmed by mass spectrometry analysis. Further in gel digestion of proteins, released by sonication and subsequently resolved by SDS PAGE identified additional proteins of the *B. anthracis* spore (Table 5.2). As with the direct digestion of the proteins released from spores by sonic disruption, some of the proteins have been reported previously in specific studies of the *B. anthracis* exosporium. A small number of proteins have not been previously identified in the *B. anthracis* spore; these are a putative lipoprotein (BA2041), hypothetical proteins (BA1021, BA5144), an adhesion lipoprotein (BA3189), a menaquinol-cytochrome c reductase, iron-sulfur subunit (BA1544) and an alkyl hydroperoxide subunit C (BA0345).



Gene Identification	Protein	Peptide sequences <sup>1</sup>	Comments
BA3594	CspB Cold shock protein B	IEVEGGED FKTLEEGQE MQNGKVKWFNSEKGF SAIQGEGFKTLEEGQEVTF	CspB BA1629 identified from unwashed exosporium (Redmond <i>et al.</i> , 2004). Identified in the soluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004)
BA4758	Thioredoxin	GEKVKVVKVDVDENQETARQ FVLKDGKVVVDQALGYKPKKEAL	Identified in the soluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004)
BA1531	DNA-binding protein HU	IGFGNF VAEASSLSKDKATKA IEIAASKVPAFKPGKALK	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). <i>B. subtilis</i> homologue Hsb is a coat protein (Lai <i>et al.</i> , 2003)
BA4499	Superoxide dismutase	AKHELNLPYAY EVTSTPNQDSPLTEGKTPVI	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Superoxide dismutase BA1489, reported as exosporium protein (Steichen <i>et al.</i> , 2005)
BA5636	Phosphate acetyltransferase	FTTVKEKVQGGKIS EAGNIGYKIAQRLGNF	Present in the soluble and insoluble fraction of <i>B. anthracis</i> spores and induced in the final stage of sporulation ("wave 5", Liu <i>et al.</i> , 2004)
BA4659	SpoVID dependent coat assembly factor SafA/ExsA	<b>MKIHIVQKGDTL<sup>2</sup></b> DTLKQTNTQLSNPDLIM <b>MKIHIVQKGDTLWKLAKKYG<sup>2</sup></b>	ExsA identified in the <i>B. cereus</i> exosporium (Bailey-Smith <i>et al.</i> , 2005)
BA1987	SASP	AQEFG AALDQM SVGGEITKR AEQQLGGGYTR	Isolated from the exosporium and soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004)
BA2292	Hypothetical protein	ILVNGQKVIKNISF VQGKKYRIDIVPVGNETPI	Present in the soluble and exosporium fraction of <i>B. anthracis</i> spores and identified as an exosporium protein (Liu <i>et al.</i> , 2004)
BA4898	SASP B sspb	AQEFG KLAVPGAE SVGGEITKR AEQQLGGFQK	Isolated from the exosporium and soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Identified as a spore coat protein (Lai <i>et al.</i> , 2003)

**Table 5.2** <sup>1,2</sup>Proteins identified from pepsin digests of sonicated spore supernatants.

<sup>1</sup>The peptide sequences were used to identify the *B. anthracis* protein from the *B. anthracis* Ames genome at NCBI (NC\_003997). <sup>2</sup>The peptide sequences identified were not present in *B. anthracis* Ames strain proteins, but were identified from *B. anthracis* Sterne (NC\_005945)

Gene Identification	Protein	Peptide sequences <sup>1</sup>	Comments
BA3920	Hypothetical protein	WKVADEQEQTIANL RLEQAQKGLDGGAVSDMA GERLEQAQKGLDGGAVSDMA EQAQKGLDGGAVSDMA	Isolated from the exosporium and soluble and insoluble fraction of <i>B. anthracis</i> spores and induced in the final stage of sporulation ("wave 5", Liu <i>et al.</i> , 2004).
BA3127	SASP	AQEFQ SVGGEITKR AEQLGGGVTR	Isolated from the exosporium and soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Identified in the spore coat but suggested it was unlikely to be a spore coat protein (Lai <i>et al.</i> , 2003)
BA2013	General stress protein dpS	FTLHEKFEEF YLATSSVNEGTSKESAE	Isolated from the exosporium and soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004).
BA4268	Phosphocarrier protein HPr ptsH-1	MEKIFKVTSDSGIHARPATL	Identified in the exosporium and soluble fractions of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004)
BA4182	Pyruvate dehydrogenase PdhC	VVEIPSPVKGKVLLEVL GIGRIAEPVVKNGEIVA IAADTDKGLLVPVVKDTD	Isolated from the exosporium and soluble and insoluble fraction of <i>B. anthracis</i> spores and induced in the final stage of sporulation ("wave 5" Liu <i>et al.</i> , 2004).
BA2162	Hypothetical protein	LVGKGFNPTIAHY	Exosporium protein denoted 'BxpA' (Steichen <i>et al.</i> , 2003)
BA0267	GroEL	RALEEPVRQ LETGIVDPAKVTRSALQN AVVVADKPEPNAPAMPDMG	Identified in unwashed exosporium (Redmond <i>et al.</i> , 2004). <i>B. cereus</i> GroEL removed by salt washing (Charlton <i>et al.</i> , 1999; Todd <i>et al.</i> , 2003). Isolated from the exosporium, soluble and insoluble fractions of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Isolated from spore coat fractions (Lai <i>et al.</i> , 2003). Component of the <i>B. anthracis</i> UK anthrax vaccine (Whiting <i>et al.</i> , 2004)
BA3129	Hypothetical protein	FGQFHMKKAQAGVTL MNHTGDEDLKKFLEGL GILSGNPQNEPLHYGEVF LRLSKKKGWVPPPLHVR LAAQGAISHQVF	Present in the soluble fraction of <i>B. anthracis</i> spores and induced in the final stage of sporulation ("wave 5" Liu <i>et al.</i> , 2004)
BA5580	Fructose biphosphate aldolase Fba-2	KEMLNKALEGKYAVGQF IDASHHPFEENVVETTKQVVE	Identified in the exosporium, soluble and insoluble fractions of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Fba-1 component of the <i>B. anthracis</i> UK anthrax vaccine (Whiting <i>et al.</i> , 2004)

Table 5.2 continued

Gene Identification	Protein	Peptide sequences <sup>1</sup>	Comments
BA4539	DnaK	PFISAAGAAGPLHLEL	Identified in the exosporium, soluble and insoluble fractions of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004)
BA0108	Elongation factor Tu	IIQLPEGTE FRKLLDQAQAGDNIGAL	Identified in unwashed exosporium (Redmond <i>et al.</i> , 2004). Identified in the exosporium, soluble and insoluble fractions of <i>B. anthracis</i> spores and identified as an exosporium protein (Liu <i>et al.</i> , 2004).
BA0887	EA1	ALDADNDGVVNYGSKLSGKD NAGDVKLGDVTVSQTSDSAL	Identified in washed and unwashed exosporium (Redmond <i>et al.</i> , 2004). Isolated from the exosporium fraction (Liu <i>et al.</i> , 2004). Isolated from spore coat fractions (Lai <i>et al.</i> , 2003)

161 **Table 5.2 continued**

Gene Identification	Protein	Peptide sequences <sup>1</sup>	Comments
BA2041	Oligopeptide ABC transporter, oligopeptide-binding protein, putative	AFVNAMEGLYR ETSTGVNLYDSDQADR VILSSEFVDK GIANVILNDGSLPAYFLVPEK ILIGQDAAISPTYQQSTAYLEKPYVK	Not identified by previous proteomics studies. Gene induced in "wave 2" of sporulation (Liu <i>et al.</i> , 2004)
BA3668	Glycosyl hydrolase family 18	LLVENFTITK FITNILQTAEK ISQTYNVPLASLAK GNNYYVQPGDSLVR AVESLAYLQPSTPIK	SleL orthologue Isolated from the exosporium and insoluble fraction of <i>B. anthracis</i> spores and identified as an exosporium protein (Liu <i>et al.</i> , 2004). Homologue of the <i>B. subtilis</i> spore coat protein YaaH (Lai <i>et al.</i> , 2003).
BA5364	Enolase	VQLVGDDLFVTNTQK AIVPSGASTGEHEAVELR	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Anthrax spore protein reactive with human sera (Kudva <i>et al.</i> , 2005). <i>B. subtilis</i> enolase associated with wild type spores (Kuwana <i>et al.</i> , 2002). Immunoreactive component of the <i>B. anthracis</i> UK anthrax vaccine (Whiting <i>et al.</i> , 2004). Adjacent and near neighbours all found in the exosporium, soluble and insoluble fractions BA5365, 5367, 5369, 5376 Liu <i>et al.</i> , 2004)
BA0108	Translation elongation factor, Tu	LLDQAQAGDNIGALLR ALQGEADWEAK TTLTAAITTVLAK	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004).
BA2075	SPFH domain/band 7 family protein	VM(Ox)QPGLNLLPIVDR	Isolated from the soluble and insoluble fraction of <i>B. anthracis</i> spores. Gene located near alanine racemase BA2979, 2068 stage II sporulation protein similar to stomatin-like protein. The gene is induced in the final stages of sporulation ("wave 5", Liu <i>et al.</i> , 2004).
BA3964	Translation elongation factor, Ts	DVAMHIAAVNPK TDADAFGAYLHMGGGR IGVLTVLEGSTDEAAAK	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). Identified from unwashed exosporium (Redmond <i>et al.</i> , 2004)
BA5363	Phosphate acetyltransferase	GISIVLPEGTDER	Present in the soluble and insoluble fraction of <i>B. anthracis</i> spores and induced in the final stage of sporulation ("wave 5", Liu <i>et al.</i> , 2004)
BA1021	Hypothetical protein	LDALPDAIK FIGHLLDPSER LPGQNNFPLLVDHTSR	Not identified by proteomics studies, induced in "wave 4" of sporulation (Liu <i>et al.</i> , 2004)
BA3446	Oxidoreductase, aldo/keto reductase family	VEEGPELVEAIK WDLQNGVITIPK	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). 79% identity to <i>B. subtilis</i> YtbE

**Table 5.3. Proteins identified from in-gel digestion of sonicated spore supernatants.<sup>1</sup>**

<sup>1</sup>The peptide sequences were used to identify the *B. anthracis* protein from the *B. anthracis* Ames genome at NCBI (NC\_003997).<sup>2</sup> Two possibilities for protein identification.

Gene Identification	Protein	Peptide sequences <sup>1</sup>	Comments
BA0345	Alkyl hydroperoxide reductase subunit C	IEYIMIGDPTR	Not identified from proteomics studies (Liu <i>et al.</i> , 2004). 79% homology to <i>B. subtilis</i> AhpC
BA0106	30S ribosomal protein S7	LANEILDAANNAGASVK	Identified from the soluble and insoluble fraction of <i>B. anthracis</i> spores
BA5641	Hypothetical protein	QATVVMTYER GSSLGTQSYTGIIIEAAGR	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores and identified as an exosporium protein (Liu <i>et al.</i> , 2004). 79% identity to <i>B. subtilis</i> spore coat protein YwdL (GerQ)
BA1234 <sup>2</sup>	ExsY (Spore coat protein Z-1)	AGAPFEAFAPSANLTSCR	Identified in the exosporium, soluble and insoluble fractions of the <i>B. anthracis</i> spore and identified as an exosporium protein (Liu <i>et al.</i> , 2004). Identified from spore coat fractions (Lai <i>et al.</i> , 2003). Isolated from washed and unwashed exosporium, denoted 'ExsY' (Redmond <i>et al.</i> , 2004)
BA1238 <sup>2</sup>	CotY (Spore coat protein Z-2)	AGAPFEAFAPSANLTSCR	Identified in the exosporium, soluble and insoluble fractions of the <i>B. anthracis</i> spore and identified as an exosporium protein (Liu <i>et al.</i> , 2004). Identified from spore coat fractions (Lai <i>et al.</i> , 2003), denoted 'CotY' (Redmond <i>et al.</i> , 2004)
BA4266	Hypothetical protein (Spore coat protein alpha, cot-alpha)	NTFTGILR VCPTDIVAIAI MFGSFGCCDNFR	Identified in the exosporium, soluble and insoluble fractions of the <i>B. anthracis</i> spore identified as an exosporium protein (Liu <i>et al.</i> , 2004) Spore coat protein (Kim <i>et al.</i> , 2004)
BA1531	DNA binding protein HU	VQLIGFGNFEVR	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). <i>B. subtilis</i> homologue Hsb is a coat protein (Lai <i>et al.</i> , 2003)
BA0251	Putative lipoprotein	NEEGVFVLTALNK	Isolated from the exosporium fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). 55% identity to YdcC a spore coat protein identified by Kuwana <i>et al.</i> , 2002)
BA3189	Adhesion lipoprotein	VSEGVEAIYLETK NADNYVAELQK ETNVPIAGTIFDLSGK	Not identified by previous proteomics studies (Gene induced in "wave 4" of sporulation, Liu <i>et al.</i> , 2004).
BA5144	Hypothetical membrane spanning protein	NCGATDGVLAIFPFLNSK	Not identified from proteomics studies gene induced in the final stages of sporulation ("wave 5" Liu <i>et al.</i> , 2004).

Table 5.3 continued

Gene Identification	Protein	Peptide sequences <sup>1</sup>	Comments
BA0640	Amino Acid ABC transporter	NPSTGQVEGFDVDVAK EVDFADVVK IFTDEPYGLAVQK	Isolated from the exosporium fraction of <i>B. anthracis</i> spores (Liu et al., 2004) Homologue of <i>B. subtilis</i> spore coat protein YckK, Lai et al., 2003
BA1544	Menaquinol-cytochrome c reductase, iron-sulphur subunit	GTPPLAPLDVYESK	Not identified from proteomics studies (Liu et al., 2004)
BA5547	ATP synthase subunit B	VEAQELIER	Isolated from the soluble and insoluble fractions of the <i>B. anthracis</i> spore (Liu et al., 2004). 85% identity to <i>B. subtilis</i> AtpD
BA0557	SPFH domain/band 7 family protein	ADADLSYELQQAK AEPLSLLNYK	Identified from the soluble fraction of the <i>B. anthracis</i> spore (Liu et al., 2004). 63% identity to <i>B. subtilis</i> YuaG
BA5548	ATP synthase subunit C	IQEVVASIAQGSK	Isolated from the soluble and insoluble fractions of the <i>B. anthracis</i> spore (Liu et al., 2004)
BA4154	cytochrome c oxidase, subunit II	IVTSSQDLVIPTTGK	Identified from the exosporium soluble and insoluble fractions of <i>B. anthracis</i> spores (Liu et al., 2004). 53% identify to <i>B. subtilis</i> CtaC
BA0715	phosphate ABC transporter, phosphate-binding protein, putative	VDNLTVQQVQDIFTGK	Not identified by previous proteomics studies. Gene induced in "wave 3" of sporulation (Liu et al., 2004). 44% identity to <i>B. subtilis</i> PstS
BA5547	ATP synthase subunit B	TVAMMSSTDGLVR VFNVLDAIDLGEVPADVR QAPAFEELSTK VEILETGK VVDLLAPYIK FTQAGSEVSALLGR ALSPEIVGEEHYEVAR YDDLPEDAFR LVGGIEEVIENAK	Identified from the exosporium, soluble and insoluble fractions of the <i>B. anthracis</i> spore (Liu et al., 2004)

Table 5.3 continued.

Gene Identification	Protein	Peptide sequences	Comments
BA4229	maltosaccharide ABC transporter, maltosaccharide-binding protein, putative	FTDSSIEAQTYNGK GIIGESGGPAADGLFNEGK	Isolated from the exosporium, soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004). 50% identity to <i>B. subtilis</i> YvfK
BA0887	EA1	GAITGKPDGTYGPTESIDR ILNLPVDENAQPSFK DFALNSQNLVVGEK VYSDPENLEGYEVESK	Identified in washed and unwashed exosporium (Redmond <i>et al.</i> , 2004) amounts varied. Isolated from the exosporium fraction (Liu <i>et al.</i> , 2004). Identified from spore coat fraction (Lai <i>et al.</i> , 2003). Reported to be a 'persistent' contaminant (Steichen <i>et al.</i> , 2004)
BA0267	GroEL	FGSPLITNDGVVIAK FGSPLITNDGVVIAK AVVAVEELK VGNDGVITLEESK ENTTVVEGVGSSTEQIEAR	Identified in unwashed exosporium (Redmond <i>et al.</i> , 2004). <i>B. cereus</i> GroEL removed by salt washing (Charlton <i>et al.</i> , 1999; Todd <i>et al.</i> , 2003). Isolated from the exosporium, soluble and insoluble fractions of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004)
BA5550	ATP synthase F1, delta subunit	NVVDEDLLGGIK	Isolated from the soluble and insoluble fraction of <i>B. anthracis</i> spores (Liu <i>et al.</i> , 2004), 47% identity to <i>B. subtilis</i> AtpH

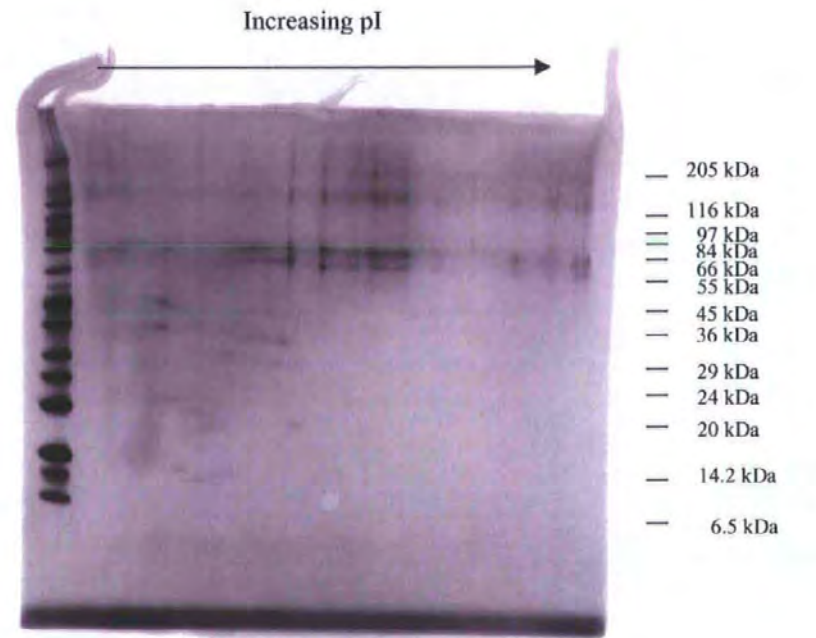
Table 5.3 continued.

Two dimensional (2D) electrophoresis was conducted on samples in order to identify a larger number of proteins; however this was difficult due to the low concentrations of the proteins released. Although confident identification of the proteins released and resolved by 2D-SDS PAGE was not possible, of note is the large number of acidic proteins. Furthermore, in comparison to a closely related *Bacillus cereus* strain NTCC 11143 a significant proportion of these proteins were not present suggesting differences in the proteins expressed by these two organisms (Figures 5.5-5.6).

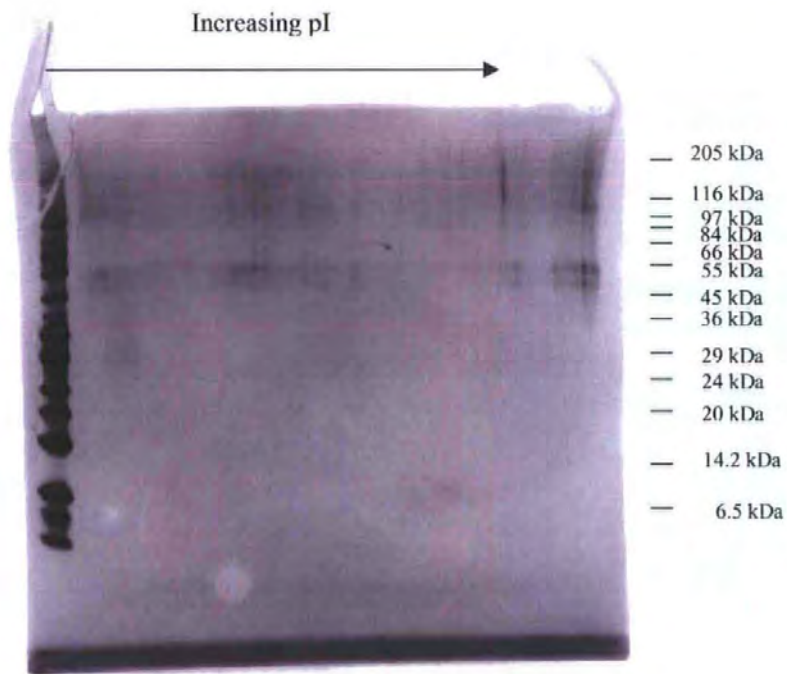
#### **5.2.4 EA1 as a detection target**

As shown in Figure 5.7, the sonication of *B. anthracis* (UM23C12 and SM91, an EA1 deletion mutant) spores and subsequent visualisation using SDS-PAGE showed that a wide range proteins of similar sized proteins were released although a number of lower molecular weight species were more predominant within SM91. As a further method of identifying proteins exposed on the spore surface, UM23C12 and SM91 spores were biotinylated and the proteins released after sonication were bound to and eluted from streptavidin coated beads. Analysis of biotinylated proteins by western blot revealed the presence of an approximately 97 kDa species identified as EA1 by nano LC-MS/MS. Attempts to identify the low molecular weight proteins in SM91 have been unsuccessful.



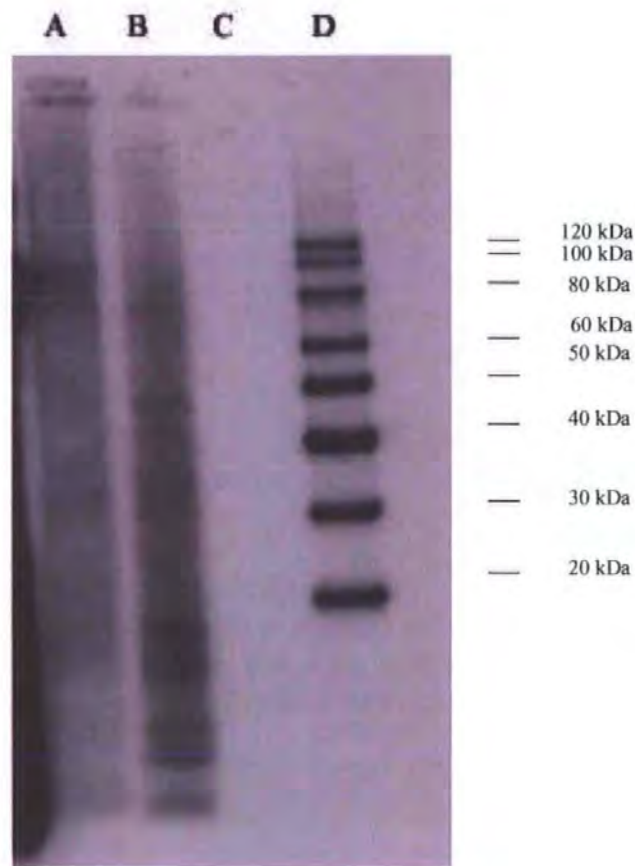


a)



b)

**Figure 5.5** Analysis of a) *B. anthracis* UM23Cl2 and b) *B. cereus* 11143 spores proteins by 2-dimensional electrophoresis. Spores were sonicated and the resultant supernatant removed and concentrated. 50 µg of sample was loaded and separated on an ZOOM IEF strip (pH 3-10, Invitrogen) and then by SDS-PAGE on 4-12% ZOOM gels. Proteins were visualised using a SilverQuest stain (both Invitrogen).



**Figure 5.6** Detection of biotinylated spore proteins from the surface of UM23C12 (pXO1<sup>+</sup>/pXO2<sup>+</sup>) and SM91 (pXO1<sup>+</sup>/pXO2<sup>+</sup>/eag<sup>-</sup>) *B. anthracis* spores. Spores were biotinylated and sonicated, the supernatants from the sonicated spores were removed and the released proteins separated by SDS-PAGE on 4-12% Bis-Tris gels (Invitrogen) and blotted onto a PVDF membrane (Invitrogen) Lane loading was as follows; A) *B. anthracis* UM23C12 spores, B) *B. anthracis* SM91 spores, C) Sonicated non-biotinylated UM23C12 spores, D) MagicMark™ Western Standard (Invitrogen)



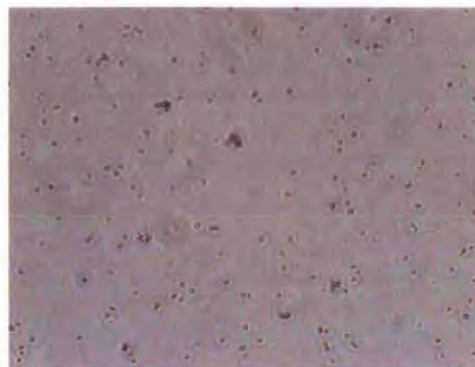
#### 5.2.4.1 Evaluation of the removal of EA1 following Urografin purification of *B. anthracis* spores

Williams and Turnbough (2004) reported that EA1 could be removed from spores following Urografin purification and three subsequent wash steps of *B. anthracis* Sterne spores, deduced from the absence of a strong band at the expected molecular weight. To assess if small amounts of EA1 were still present, this purification procedure was followed and western blot analysis using a monoclonal  $\alpha$ -EA1 antibody was used to determine the presence of EA1 in purified sonicated spores. The results revealed that although the Urografin purification of the spores removed a small proportion of the EA1 present, it was still detectable by western blot analysis; furthermore, this band was still visible when proteins were separated and visualised by SDS PAGE (data not shown). This finding prompted the study of the nature of the association of the EA1 with the spore. Therefore, the visualisation of the binding of FITC labelled antibody was undertaken. Surprisingly, in this case the results suggest that these antibodies bound to whole spores and this was still observed after purification. Representative results are shown in Figure 5.8. As would be expected no binding of FITC to the spore in the absence of antibody was observed.

To ensure that the observed result was not due to the presence of another protein that contained an epitope that was similar to that recognised by the  $\alpha$ -EA1 antibody, direct pepsin digestion and subsequent LC-MS/MS of the proteins released from purified *B. anthracis* UM23C12 by sonication was undertaken. This confirmed the presence of EA1 within the spores. However, the question as to whether EA1 was present in different amounts in different strains needed to be assessed, as the Sterne strain had been used previously (Williams and Turnbough, 2004). Thus, two monoclonal antibodies raised against purified EA1 were assessed for the ability to detect  $\gamma$ -irradiated wildtype type Ames,  $\gamma$ -irradiated Sterne and the avirulent UM23C12 *B. anthracis* strains. Using a direct assay format no detection of whole spores was observed with  $\alpha$ -EA1 mAb 1 or  $\alpha$ -EA1 mAb 2 with UM23C12 spores. However, in the case of *Bacillus anthracis*  $\gamma$ -irradiated (Wildtype) Ames strain the  $\alpha$ -EA1 mAb 1 showed a 5-fold decrease in assay sensitivity compared to anti-EA1 mAb 2. In the case of  $\gamma$ -irradiated Sterne spores the results showed a slight increase in assay sensitivity compared to results obtained using the  $\gamma$ -irradiated Ames strain. Analysis of sonicated spore supernatants and S-layer proteins demonstrated the presence of EA1 and its homologues in various *Bacillus* species in spore preparations, although the amounts and the molecular weights of the proteins vary in each species (Figure 5.9).



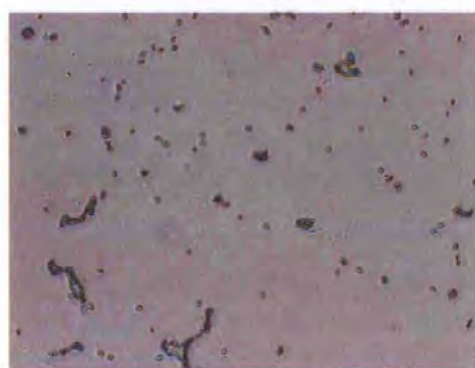
a)



b)



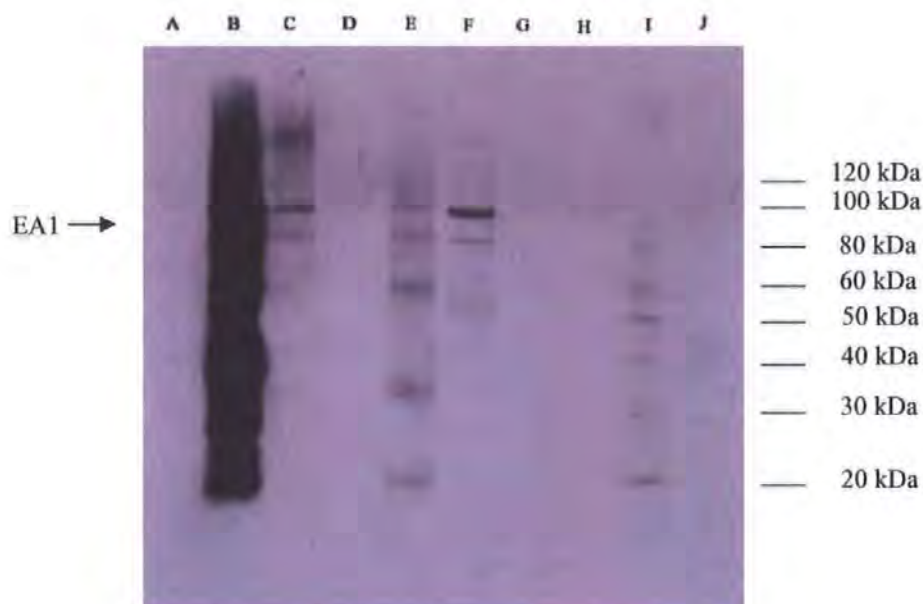
c)



d)

**Figure 5.7** Evaluation of the binding of a FITC-labelled anti-EA1 mAb to washed and Urografin purified and washed *B. anthracis* UM23Cl2 spores. A) *B. anthracis* spores washed 10x viewed by fluorescence microscopy (x1000), B) *B. anthracis* spores washed 10x viewed by phase contrast microscopy (x1000), C) *B. anthracis* spores washed 10x ,Urografin purified and washed 3x viewed by fluorescence microscopy (x1000), D) *B. anthracis* spores washed 10x Urografin purified and washed 3x, viewed by phase contrast microscopy (x1000).





**Figure 5.8 Detection of proteins from sonicated spore supernatants by mAb 2 (30s exposure).** Approximately 20  $\mu\text{g}$  of protein was loaded onto a 10% Bis-Tris (Invitrogen), separated by SDS-PAGE and blotted onto a nitrocellulose membrane (Invitrogen). Blots were probed with anti-EA1 mAb at 10  $\mu\text{g}/\text{ml}$  and visualised using a rabbit anti-mouse HRP at 1: 10000 dilution. A) *B. cereus* 9946 , B) *B. cereus* 11145, C) *B. cereus* 11143, D) *B. pumilus*, E) *B. anthracis* UM23Cl2, F) *B. anthracis*  $\gamma$ -irradiated Ames , F) *B. thurigiensis* var. *kuraskai*, G) *B. thurigiensis* var. *israelensis*, H) *B. pumilus*, I) MagicMark™ Western Standard, J) Ovalbumin control

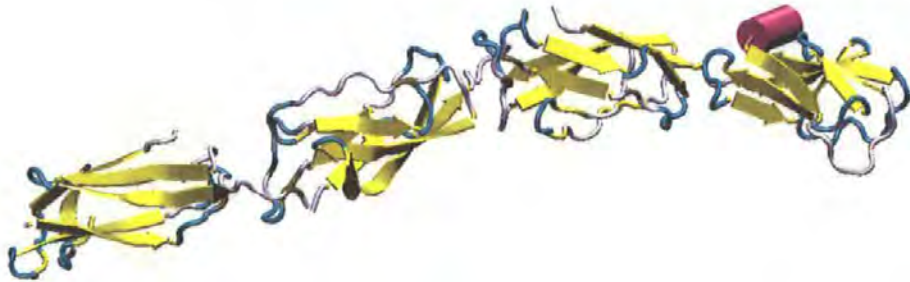
## 5.2.5 Modelling of putative detection targets and identification of possible surface located unique protein signatures

### 5.2.5.1 EA1

Modelling was kindly conducted by Dr R. Balhorn at the Lawrence Livermore National Laboratories (LLNL), Livermore CA, USA. The EA1 protein was shown to exhibit a low level (10% - 15%) of sequence identity to a number of proteins whose structures were accessible at the Protein Data Bank (PDB). These structures were as follows; 1CWV\_A (15%), 1F00\_I (14%), 1BU8\_A (10%) and 1G0D\_A (25%). Even though a low level of sequence identity was observed, sequence-structure alignments produced by various techniques, yielded hits to these PDB templates. The level of sequence similarity between two first templates (1CWV and 1F00) was ~32% (however, in PDB only the C-terminal fragments of these two templates are provided). Both are multi-domain structures

composed from a few "All  $\beta$ " structural domains (middle part of the protein) and one " $\alpha + \beta$ " domain (the C-terminal part of the protein). Each one of these domains was approximately 100 aa long.

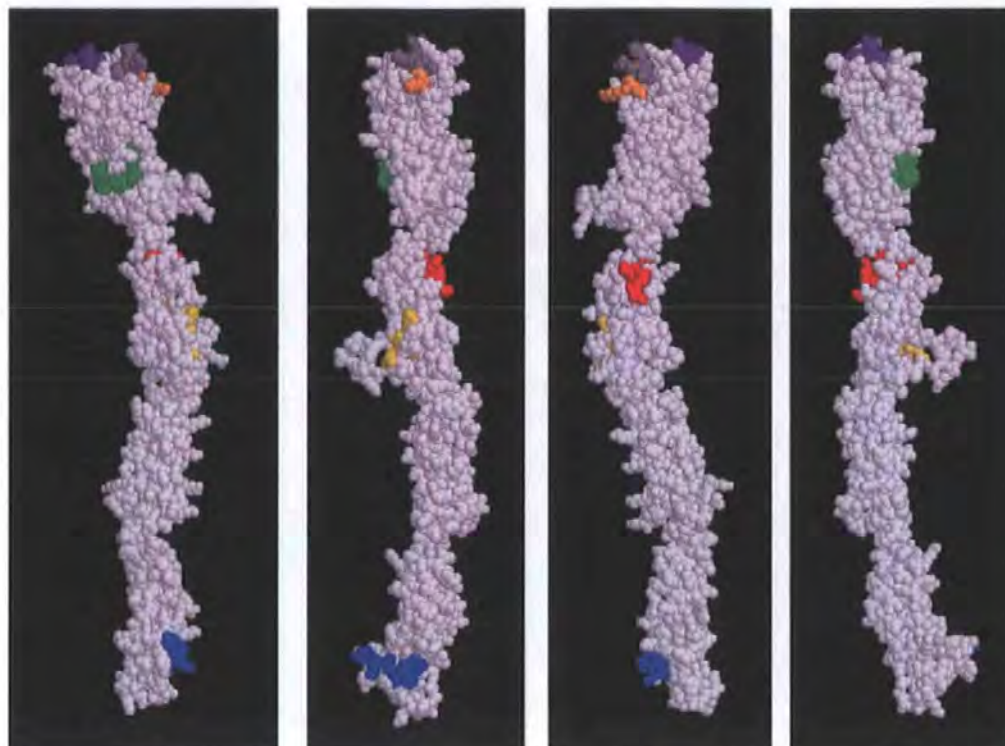
Using the Structural Classification of Proteins (SCOP), the fold of one domain was classified as an immunoglobulin-like  $\beta$ -sandwich (7 strands in 2 sheets) similar to that found in invasin/intimin cell-adhesion fragments. The second fold was observed to be a C-type lectin-like domain similar to that found in enteropathogenic *E. coli* intimin. Sufficient sequence and structural identity were present for the most, but not all, of the *B. anthracis* EA1 molecule. As a result, LLNL were able to generate a homology model for residues 302-803. Since the entire protein is 862 amino acids long, the model is missing ~300 amino acids on the amino terminus and 59 amino acids on the carboxy-terminus of the protein. After creation of the initial model this structure was subsequently refined. The final model containing four of the structural domains is shown in Figure 5.9



**Figure 5.9 Homology model of *B. anthracis* EA1 showing the four structural domains Residues 302-803 (Conducted by Dr R. Balhorn, LLNL).**

Following the identification of unique protein signatures for EA1, these were mapped onto the final structure to identify those signatures that should be accessible for binding by an appropriate recognition element (Figure 5.10). In total, a set of ten unique regions was identified (Figure 5.11). Of these eight, were located within the region of the protein that was modelled. Regions that could not be mapped onto the protein structure were located in the first 300 amino-terminal amino acids.





**Figure 5.10** Four views of EA1 with the unique peptide signatures mapped onto the surface and identified as coloured amino acids. (Conducted by Dr R. Balhorn, LLNL).

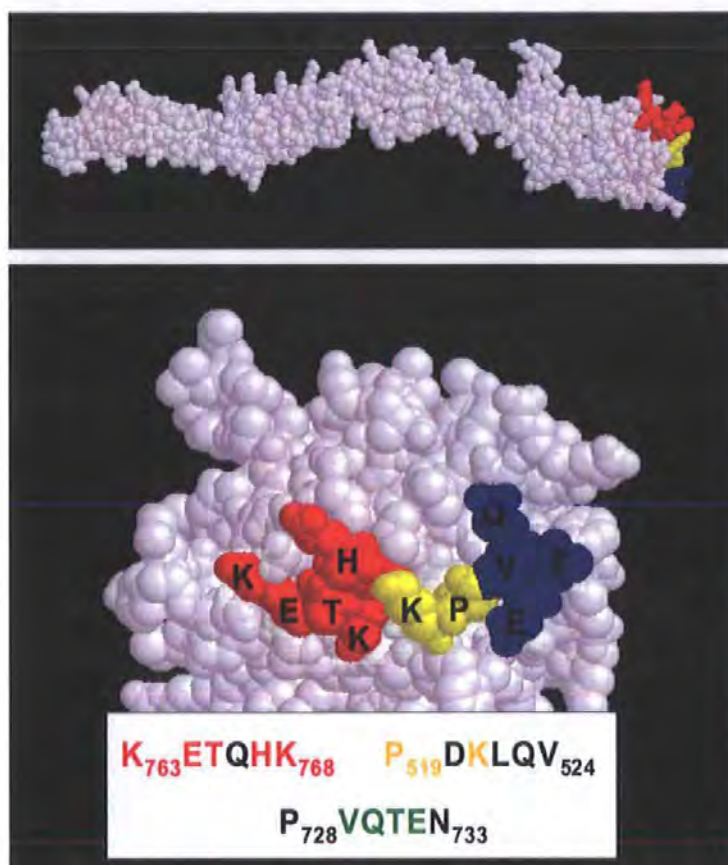
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GKSFDPVPAGHWAEGSINYLVDKGAI TGKPDGTYGPTESIDRASAAVIFTKILNLPVDENA
QPSFKDAKNIWSSKYIAAVEKAGVVKGDGKENFYPEGKIDRASFASMLVSAYNLKDKVNGE
LVTTTFEDLLDHWGEEKANILINLGI SVGTGGKWEPNKSVSRAEAAQFIALTDKKYKGDNA
QAYVTDVKVSEPTKLTLTGTGLDKLSADDVTLEGDKAVAI EASTDGTSAVVTLGGKVAPNK
DLTVKVKNQSFVTKFVYEVKKLAVEKLTFFDDDRAGQAIAPKLNDEKGNADVEYLNLANHDV
KVVANNLDGSPANIFEEGEATSTTGKLAVGIKQGDYKVEVQVTKRGGLTVSNTGII TVKNL
DTPASAIKNVVFALDADNDGVVNYGSKLSGKDPALNSQNLVVGEKASLNKLVATIAGEDKV
VDPGSISIKSSNHGII SVVNNYITAEAAAGEATLTIKVGDTVTKDVKFKVTTDSRKLVSVKAN
PDKLQVVQNKTLPVTFVTTDQYGDPPGANTAAI KEVLPKTVVVAEGGLDVVTTDSGSIGTK
TIGVTGNDVGEGVHPQKNGATLGSLYVNVTEGNVAFKNFELVSKVGQYQSPDTKLDLN
VSTTVEYQLSKYTSDRVYSDPENLEGEYEVESKNLAVADAKIVGNKVVVTGKTPGKVDIHLT
KNGATAGKATVEIVQETIAIKSVNFKPVQTENFVEKKINIGTVLELEKSNLDDIVKGINLT
KETQHKVRVVKSGAEQKLYLDRNGDAVFNAGDVKLGDTVVSQTSDSALPNFKADLYDTLT
TKYTDKGTLVFKVLKDK

```

**Figure 5.11** Sequence of *B.anthraxis* EA1 showing the location of the signatures with the signal peptide removed. Sequences unique to *B. anthracis* EA1 are shown in red.

Evaluation of the location of the unique regions predicted that most of the sequences identified were surface accessible. Furthermore, a number of these regions were clustered towards the C-terminus of the protein (Figure 5.12).



**Figure 5.12** Structure of EA1 carboxy-terminal domain showing three clustered unique peptide sequences. (Conducted by Dr R Balhorn, LLNL). The colours show three unique peptide sequences which together, form a cluster on the protein's surface.

### 5.2.5.2 Alanine Racemase

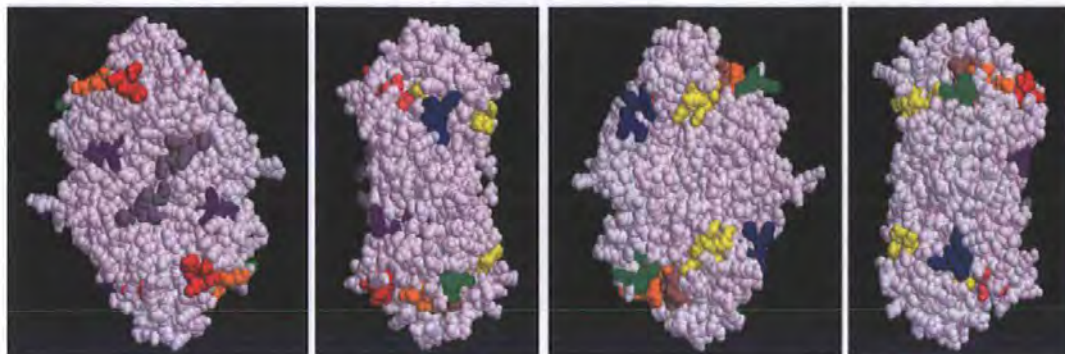
Alanine racemase was also modelled and the location of unique protein regions identified. Searches of the PDB revealed nine template molecules that had significant (13-39%) sequence identity to *B. anthracis* alanine racemase: 1SFT\_A (39%), 1I6F\_A (39%), 1EPV\_A (39%), 1B54 (16%), 1CT5 (16%), 1D7K\_A (14%), 1F3T\_B (14%), 2T0D\_A (14%) and 70DC\_A (13%). A number of iterations of the homology model were produced using 1SFT as the starting template. A model of the monomeric form of the protein was created in Figure 5.13. However, using known facts regarding the protein the final iteration



of the model was created as a homodimer. In total eleven unique regions were identified within the protein sequence and mapped onto the structure of the homology model (Figure 5.14). As was observed for EA1, several of these unique signatures were also predicted to be located within a cluster. However, as sequence data has been added to that available at NCBI these regions have been found to no longer be unique. Alanine racemase now has up to 99% identity with proteins homologues in closely related *Bacillus* species, and as such was disregarded as a target for the generation of specific recognition elements.



**Figure 5.13 Homology model of the *B. anthracis* alanine racemase monomer.**  
The location of the unique protein signatures are coloured in green.



**Figure 5.14** Space fill model of the *B. anthracis* alanine racemase dimer. View 1-4 (left to right), dimer rotated to left 90° each view. The location of the original unique protein signatures are shown as coloured amino acid side chains, however, these regions are no longer unique. (Conducted by Dr. R. Balhorn, LLNL).

### 5.2.5.3 Adhesion lipoprotein BA3189

Preliminary analysis of the BA3189 protein enabled the three dimensional structure to be modelled for the majority of the protein (highlighted in green, Figure 5.15). Unique regions of the protein were identified as mapped onto the structure. The regions 'DVMKMT', WMNIKN and LKMMKV were predicted to be surface accessible (Figure 5.16). Again these surface accessible regions could be exploited for the production of specific recognition elements.

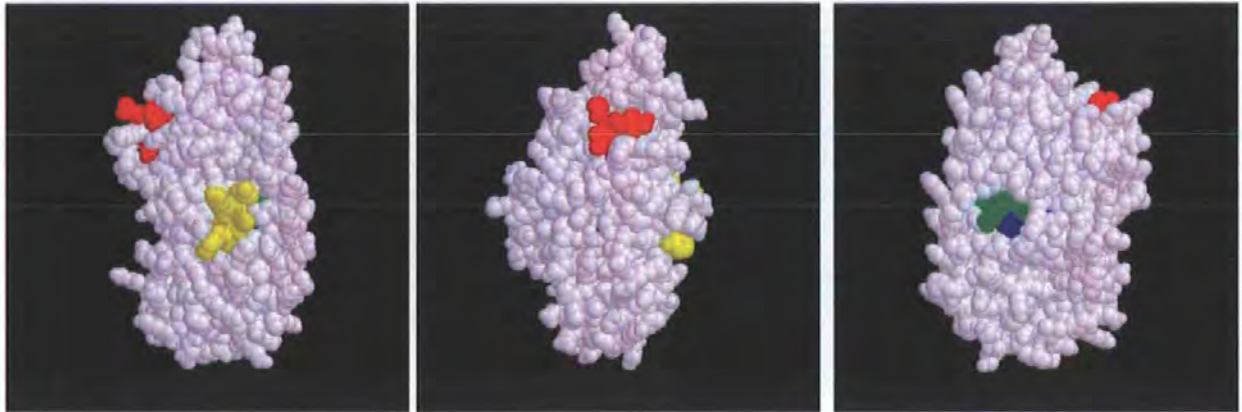
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CSSNTNGKEEGSGKLVVTTYSIIYDMVKQIGGEKVEIHSLVPIGANPHEYDPLPKDDVMKMT
DADMVLYNGLNLEEGGAWFKLLKTANKSEKDAPVYKVSEGVVAIYLETGKLEKEPDPHAWM
NIKNGILYAENVKKALIKEDPKNKEFYTKNADNYVAELQKLHDETVNRIHQIPEEKRFLLISSE
GAFKYFGKAYDIKTGYIWEINSENQGTPDQIRDVVSVIQTNKVPALFVETSVDRRSMETVSKE
TNVPIAGTIFTDSLKSGEDGDTYLKMMKVNIDTIINGLQK

```

**Figure 5.15** Unique regions in the BA3189 sequence with the signal peptide removed. The modelled portion of the sequence is in green and unique regions are shown in red.





Red, 77-82: DVMKMT  
 Blue, 140-145: PHAWMN

Green, 143-148: WMNIKN  
 Yellow, 295-300: LKMMKW:

**Figure 5.16** Space fill model for BA3189 showing predicted surface located unique regions of the protein. (Conducted by Dr. R. Balhorn, LLNL).

#### 5.2.5.4 *ExsA*

BLAST searches and sequence alignment revealed two regions that appeared to be unique to *B. anthracis* (Figure 5.17). These may represent areas that can be targeted for the production of specific recognition elements. However, to date, it has not been possible to complete modelling of the proteins and the mapping of these regions onto the protein structure.

```

1  MQKGDTLWKIAKKYGVDFDTLKQNTQLSNPDLIMPGMKIKVPSNGVQVKQHAGAGSAPP  60  B.  a
   +QKGDTLWKIAKKYGVDFDTLK+TNTQLSNPDLIMPGMKIKVPS  V +KQ  AGAGSAPP
6  VQKGDTLWKIAKKYGVDFDTLKKTNTQLSNPDLIMPGMKIKVPSKSVHMQQAGAGSAPP  65  B.  c

121 KEVQVKPQKEMQVKPQKEVQKEKLIQIEKPVEKPVVEKPSVIQKPPVIEKQKPAEKENTKF 180 B.  a
    KEVQVKPQKEMQVKPQKEVQKE+ IQ      EKPVEKPSVIQKPPVIEKQKPAEKENTKF
126 KEVQVKPQKEMQVKPQKEVQKEQPIQ----KEKPVEKPSVIQKPPVIEKQKPAEKENTKF 181 B.  c

```

**Figure 5.17** Areas of protein sequence showing differences in ExsA by comparison of *B. anthracis* (Ames) (B. a) and *B. cereus* ATCC 10876 (B. c) described by Bailey-Smith *et al.*, 2005. The regions of disparity appear to be conserved amongst all other members of the *B. cereus* group for which protein sequence data is available at <http://www.ncbi.nih.gov/> (data not shown) (*B. cereus*, *B. thuringiensis* and *B. weihenstephanis*).

#### 5.2.5.5 *BxpA*

BLAST searches and sequence alignment revealed two regions that appeared to be unique to *B. anthracis* (Figure 5.19). These may represent areas that can be targeted for the production of specific recognition elements. To date, it has not been possible to complete modelling of the proteins and the mapping of these regions onto the protein structure

```

40 ATPQNQELQYPQNPYTAPQTQEQQFQQNSYDTRPSYIEYPQNPYAAPQNQELQYPQNPYVT  99  B.  a
   ATPQ  E  YPQNPYTAPQTQEQQFQQNSYDTRP+YEYPQNPYAAPQNQELQYPQNPYVT
45 ATPQYLEADYPQNPYTAPQTQEQQFQQNSYDTRPNYIEYPQNPYAAPQNQELQYPQNPYVT 104 B.  c

```

**Figure 5.18** Areas of protein sequence showing differences in BxpA by comparison of *B. anthracis* (Ames) (B. a) and *B. cereus* G9421 (B. c). The regions of disparity appear to be conserved amongst other members of the *B. cereus* group for which protein sequence data is available at <http://www.ncbi.nih.gov/> (data not shown) (*B. cereus*, *B. thuringiensis* and *B. weihenstephanis*).

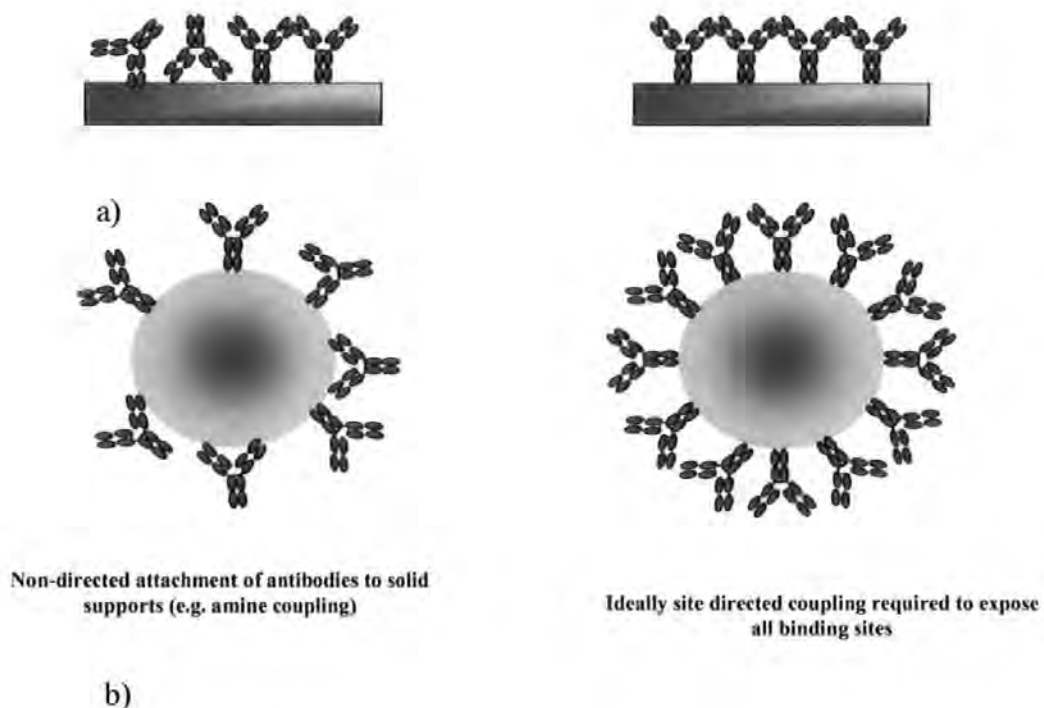
### 5.3 Discussion

The aim of this study was to elucidate structural and surface antigenic components of *B. anthracis* spores. The knowledge gained could be used in a rational design process to generate recognition elements for the specific detection of *B. anthracis*.

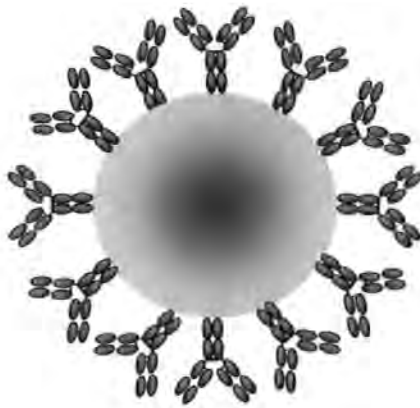
FITC labelling of *B. anthracis* spores was not successful for the identification of surface located proteins. This was probably due to the lowered efficiency of fluorescence observed with FITC than would be observed with molecules such as Alexafluor dyes. The biotinylation of spores appeared to be a promising technique for the identification of surface located proteins. This method has been used by a number of groups for the surface labelling of yeast cells (Casamova *et al.*, 1992; Alexandre *et al.*, 2000;), mammalian cells (Hurley *et al.*, 1985) and microorganisms (Masouka *et al.*, 2002; Flesch *et al.*, 1995; Mendis *et al.*, 2001; Cosma, 1997). However, in the modification and application of this method to spores it was found that the low amounts of protein, even following concentration, inhibited positive identification of all of the proteins released except EA1. Since successful use of this technique would require very large quantities of spores to be biotinylated and sonicated, it was not pursued. It is also possible that the proteins released were not suitable for mass spectrometry based identification, although as these techniques improve this method may prove valuable for the identification of surface located targets and provide more important insights into the structure of the *B. anthracis* spore.

Steichen *et al.* (2003) demonstrated that BclA was an immunodominant protein of the *B. anthracis* exosporium by evaluating a number of monoclonal antibodies by western blot analysis. In order to establish whether further immunogenic proteins exist within this surface layer that may represent markers for a detection or diagnostic assay, an immunopurification procedure (Madey *et al.*, 2000; Persaud *et al.*, 2003) was developed. The method employed utilised  $\alpha$ -species beads and a polyclonal antibody. The use of the  $\alpha$ -species beads may have aided in the elution of sufficient protein for identification compared to other methods which may reduce the number of binding sites available to antigen, as correct orientation and increased surface loading of the antibody can be achieved (Figure 5.19) (Huang *et al.*, 1996; Nisnevitch and Firer, 2001). Furthermore, the increase in the distance between the antigen binding site and the support would have

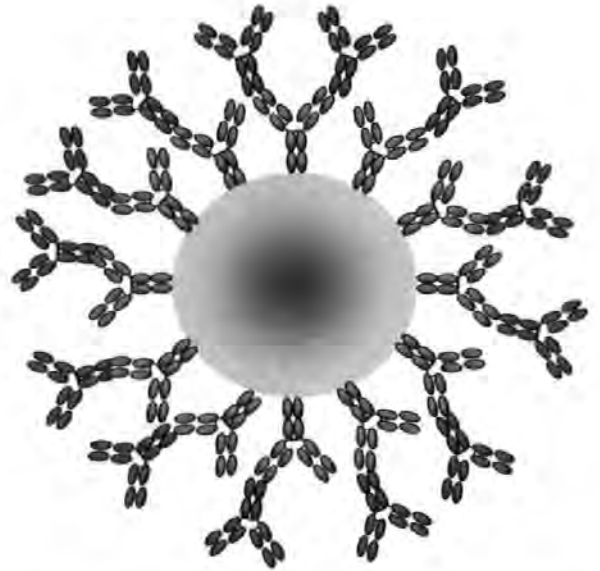
decreased the possibility of steric hindrance, as shown in Figure 5.20 (Huang *et al.*, 1996; Nisnevitch and Firer, 1999).



**Figure 5.19** Advantages of site directed antibody immobilisation on a) planar and b) spherical supports.



Anti-species beads (e.g. goat anti-rabbit)



Addition of secondary antibody (e.g. a rabbit polyclonal antibody) allowing for correct orientation of antibody

**Figure 5.20** Decreasing steric hindrance of antigen binding with the use of anti-species beads

The choice of a polyclonal antibody allowed for the identification of a greater number of targets than would have been identified if a monoclonal antibody had been used. The majority of monoclonal antibodies resulting from immunisation with a complex protein mixture, such as the exosporium, are likely to react with the most immunodominant protein species present, in this case, BclA (Steichen *et al.*, 2003). In contrast, a polyclonal antibody will recognise a range of proteins that are capable of eliciting an immune response.

From the results obtained, a number of immunogenic exosporium proteins were identified (BA2332, ExsY, ExsF, CotY, Cot- $\alpha$ , alanine racemase, CotB and a second CotB homologue). The detection of multiple proteins within single bands suggested some interactions of these proteins that may exist within the exosporium.



BA2322 was first shown to be present experimentally in unwashed exosporium preparations by Redmond *et al.* (2004), although whether this was a true structural component of the exosporium or an adsorbed protein was unclear. Steichen *et al.* (2005) cites BA2332 in a list of putative basal layer proteins. From this work BA2332 was identified as an immunogenic component of the exosporium and as such has been designated ExsM.

ExsY, ExsK and CotY were also found to be immunogenic and the presence of ExsY and CotY in a high molecular weight complex, at 250 kDa was also observed. It has been suggested that ExsY and CotY form multimeric complexes with ExsF (ExsFA/ BxpB) and BclA, as well as existing in a monomeric form (Redmond *et al.*, 2004; Sylvestre *et al.*, 2005). However, in this study no sequences derived from ExsF (ExsFA/ BxpB) were identified. The absence of peptides from BclA is unsurprising as this protein is not amenable to mass spectrometry based methods of identification due to its hydrophobic nature and relatively few tryptic cleavage sites.

A second lower molecular weight complex was observed at 40 kDa containing ExsK, ExsY and CotY possibly with alanine racemase or an as yet unidentified protein. The presence of alanine racemase, an enzyme involved in germination, has been described by a number of groups, within the exosporium from both *B. anthracis* (Liu *et al.*, 2004; Redmond *et al.*, 2004) and *B. cereus* (ATCC 10876) (Todd *et al.*, 2003) spores. It is not known whether the presence of this enzyme is due to a close association with the exosporium or whether it functions as a structural component as part of the regulatory mechanism for spore germination (Steichen *et al.*, 2003; Todd *et al.*, 2003; Redmond *et al.*, 2004). The presence of alanine racemase within the multimeric ExsK, ExsY and CotY complex may suggest a role for these proteins in maintaining this enzyme within the exosporium. Recent work conducted by Sylvestre *et al.* (2005) reported the detection of proteins at approximately 37 kDa using antibodies generated from  $\Delta$ BclA exosporium or  $\alpha$ -ExsFA antibody. These proteins were not detected using the same antibodies when  $\Delta$ ExsFA spore extracts were probed. Thus these as yet unidentified proteins were hypothesised to require the exsFA gene for assembly within the spore. It is possible that the bands identified at 37 kDa from the Sylvestre study contain the proteins identified in the 40 kDa band from this study, accounting for errors in the determination of molecular weight. Alternatively ExsFA may be required for the assembly of one or a number of exosporium proteins which may include ExsK, ExsY and / or CotY. The others may



subsequently form as a complex containing alanine racemase. The alanine racemase could then be released upon degradation of the exosporium. Thus, ExsK, ExsY and CotY may be present in multimeric complexes and require the ExsFA gene for assembly, although this would have to be confirmed experimentally. The requirement for ExsFA for the formation of a complex of the exosporium proteins containing alanine racemase rather than a reduction in the level of this enzyme may also account for the slower germination rate observed for the  $\Delta$ ExsFA (BxpB) mutant compared to wildtype *B. anthracis* spores (Steichen *et al.*, 2005).

The presence of CotB1 has been reported previously (Redmond *et al.*, 2004; Liu *et al.*, 2004). The results from this work suggest that it is also immunogenic. However, experimental identification of a second CotB homologue within unwashed Ames exosporium has not been determined experimentally to date. Thus, this protein has been designated CotB2. Sequences for these proteins within the annotated Ames genome sequence are either degenerate or absent. However, the entire protein encoding sequence is present within the *B. anthracis* Ames genome sequence and from this work the protein has been shown to be present experimentally, thus re-annotation of this area may be necessary. The sequences for the two CotB proteins are present within *B. anthracis* Ames A2012 and *B. anthracis* Sterne (BAS0340 and BAS0340). In both strains CotB1 and CotB2 are flanked by a DNA binding domain and a hypothetical protein similar to *B. subtilis* YrwJ and evidence suggests that this is conserved within the Ames genome. The suggested sequences of these proteins are denoted below (Figure 5.21) and were derived from nucleotide sequence data using the Expasy translation tool (<http://www.expasy.org/tools/dna.html>). Figure 5.22 shows the current annotation of this region within the Ames genome and the suggested organisation from this work.

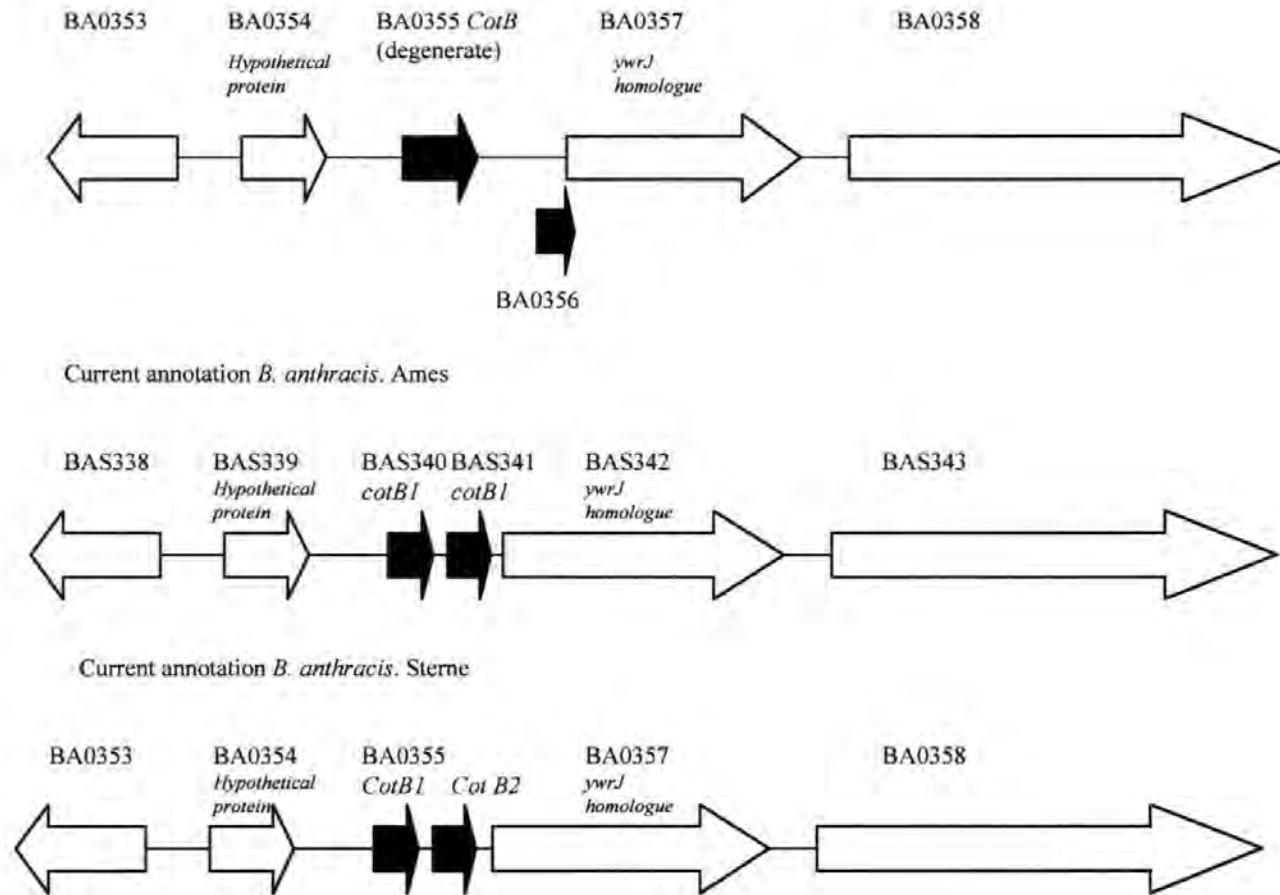
Cot B1

VSLFHCDLFDLIGSFVRVNRGGPESQRGTIISVCPDYFVLQNEKGELYYYQLSHLKSITKS  
AKECGSSDCEWEDCACAEDFEALLESFKYRWVKINRGGPEKVEGILQDVSCDFVTLIVKE  
EIIILAIKHIKSVNYNALACGESDESDDSSSKESSNNSGRARAQRQSSRGR

CotB2

LENVLCCDQIKCLVGETVKVNLRGPESRVGELVSLGKDYLTLQLPHGELVYYQLKHKVKS  
LVKKVKESKCGDCYSSCFCSDEDTFLDILKDLKYKWKINRGGPECMDGLLSEVHHGCIT  
LVNCDEVIYVINSHIKSVSQVVKCKKNEDE

**Figure 5.21 Predicted sequence of CotB1 and CotB2 from translation of the nucleotide sequence obtained from the *B. anthracis* Ames complete genome NCBI Ref NC\_003997.3 (CotB1 365687-366215, CotB2 366228-366678)**



**Figure 5.21** Suggested re-annotation of the *cotB* region of the *B. anthracis* Ames genome

The *B. anthracis* CotB homologue has 30% identity to *B. subtilis* CotB. In *B. subtilis* CotB is located in the outer spore coat (Sacco *et al.*, 1995; Kuwana *et al.*, 2002) and is present as a 46 kDa protein (CotB46), which is post-translationally modified to a 66 kDa form (CotB66), (Zilhao *et al.*, 2005). Examination of the *B. subtilis* CotB sequence reveals few cysteine residues, unlike the relatively cysteine rich CotB1 and CotB2 proteins within *B. anthracis* (Zilhao *et al.*, 2004). The cysteine rich nature of CotB within *B. anthracis* would allow for the formation of covalent crosslinks through disulphide bonds and/or have a role in binding metals (Steichen *et al.*, 2005). Therefore, the interaction of CotB with other proteins between the two species may not be the same. Chada *et al.* (2003) also describe a role for *B. subtilis* CotB in the ridge pattern formation observed in *B. subtilis* spores and implicated the role of other genes *cotM*, *cotX*, *cotY*, *cotZ* and the *cge* genes in spore morphogenesis. The proteins expressed by these genes have also been hypothesised to glycosylate proteins located on the spore surface (Roels and Losick, 1995). This group also demonstrated that CotB can form a complex with itself and CotG, a homologue of ExsB, an exosporium protein identified in *B. cereus* by Todd *et al.* (2003). No peptides derived from *B. anthracis* ExsB in the bands containing the CotB proteins were identified in this study. However, preliminary results suggest that interactions may occur between both CotB homologues and ExsF, ExsY, CotY and ExsK due to the presence of peptides derived from these proteins in a single band. However, this was difficult to ascertain due to their migration close to their predicted molecular weight. All of these proteins have however been cited as predicted putative basal layer proteins by Steichen *et al.* (2005) which may indicate that interactions between these proteins may occur.

The cot- $\alpha$  homologue was also identified as an immunogenic component of the exosporium. Kim *et al.* (2004) showed this protein to be localised to the outer spore coat and absent from the exosporium by immunogold labelling. However, Liu *et al.* (2004) describes this protein in the exosporium fraction of *B. anthracis* spores suggesting that this protein may also be isolated using exosporium extraction methods.

A number of other proteins have been described within exosporium preparations but were not identified/found as part of this study. The list of proteins identified during this study is by no means exhaustive and other proteins in, or associated with, the exosporium may be immunogenic.

Sonication of bacterial spores was shown to enhance assay sensitivity. Sonication may not only release proteins from the exosporium but from underlying layers such as the spore coat. Therefore, the number of potential antigens that could be used for detection would increase, increasing the probability of finding specific proteins or proteins with unique regions. The identification of a number of proteins from a complex mixture by direct pepsin digestion following sonic disruption was shown to extend the number of putative targets for detection. All of the proteins identified using this approach had been described previously within the *B. anthracis* spore, with the exception of *B. cereus* ExsA homologue. This protein has been shown to play an important role in the formation and assembly exosporium (Bailey-Smith *et al.*, 2004).

The use of in-gel digestion of proteins released by sonication identified further proteins of the *B. anthracis* spore. A number of these have been reported in the exosporium or coat extracts of spores (Lai *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004, Steichen *et al.*, 2004), supporting the observation that sonication removes the outer layers. However, a number of proteins have not been described previously including a putative lipoprotein (BA2041), hypothetical proteins (BA1021, BA5144) an adhesion lipoprotein (BA3189), a menaquinol-cytochrome c reductase iron-sulfur subunit (BA1544) and an alkyl hydroperoxide subunit C (BA0345). The putative lipoprotein BA2041 has some similarity to OppA, which is a predicted cell wall protein. However, possibly of note is the fact that this protein is located downstream of ExsB (BA2045) and thus may also have a function within the spore. No function of the two hypothetical proteins (BA1021 and BA5144) could be inferred by BLAST searches, although BA5144 is specific to the *B. cereus* group and BA1021 also has homologues in *Clostridium* species which may suggest some function within the spore. The BA0345 protein is an alkylhydroperoxide subunit C, a homologue of this protein has been described in *B. subtilis* spores (Casillas-Martinez and Setlow, 1997; Kuwana *et al.*, 2002). This enzyme may be speculated to have a role in the protection against oxidative stress, however this has been shown not to be the case for *B. subtilis* spores (Casillas-Martinez and Setlow, 1997). However, a possible function in *B. anthracis* as a protection mechanism against the oxidative stress encountered in the host macrophage cannot be completely discounted (Kang *et al.*, 2005).

The majority of proteins identified would be unsuitable as detection markers as they showed high identity with other members of the *B. cereus* group and surface exposed unique regions could not be found. However, on examination of sequence and structural predictions ExsA, BxpA, EA1 and BA3189 were potential candidates. The BxpA protein (BA2162) was previously identified as a component of the *B. anthracis* exosporium (Steichen *et al.*, 2003). The sequence of the ExsA protein of *B. anthracis* shares up to 91% identity with other members of the *B. cereus* group. From examination of the protein sequence, two regions were identified that showed significant differences between homologous proteins within the *B. cereus* group (Figure 5.17). Other differences observed were in the numbers of proline tandem repeats present towards the C-terminal region of the protein, but as these are merely repeated short sequences they would not be suitable as linear epitopes. The lower degree of sequence identity of this protein compared to other putative targets suggests that this could be exploited for the development of specific recognition elements. However, computer modelling would be required to ascertain if these unique regions are surface exposed. Of particular interest was the identification of an adhesion lipoprotein (BA3189). This protein shared only 94% identity to a homologue in *B. cereus* E33L a strain of *B. cereus* causative of anthrax-like disease in animals. This protein shows promise as a *B. anthracis*-specific marker and more recently has also been reported to be a novel chromosomally encoded *B. anthracis* virulence factor termed MntA expressed by vegetative cells (Gat *et al.*, 2005). Further experiments would have to be conducted to assign functions to the proteins of interest within the spore.

EA1 was investigated as a preliminary target as it was consistently identified as a component of the spore and has since been reported in a number of studies of the exosporium and spore coats of *B. anthracis* (Lai *et al.*, 2003; Redmond *et al.*, 2004; Liu *et al.*, 2004). Furthermore, as none of the proteins identified were unique, it was unlikely that traditional methods of antibody production would yield a specific detection reagent. Computer modelling predictions suggested the existence of a surface exposed unique regions for MntA and alanine racemase. However, in the case of EA1, clusters were identified that could be exploited to develop specific recognition elements that could potentially recognise unique three dimensional epitopes containing multiple unique protein signatures. Cavities on the protein surface surrounding or encompassed by the cluster also provide unique target sites.

EA1 is a vegetative cell protein; however, results from work confirms earlier reports that it is also present in spore preparations (Lai *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004) and washed wildtype Ames exosporium (Redmond *et al.*, 2004). At the time of conducting this study Williams and Turnbough (2004) suggested that EA1 was a contaminant within spore preparations. However, Western blot analysis using an anti-EA1 antibody demonstrated the presence of EA1 in spores prepared as described by Williams and Turnbough (2004), which was confirmed by mass spectrometry. This suggested a closer association than previously suggested. However, a decrease in the amount of EA1 detectable after Urografin purification of *B. anthracis* spores indicated that wash steps do remove a small proportion of this protein target. The detection of EA1 in all strains tested (UM23C12, Sterne and wildtype Ames), suggests that this observation was not purely due to the original strain utilised, UM23C12. Evaluation of two anti-EA1 antibodies by Western blot analysis suggested EA1 exists in truncated forms and/or a range of proteins may be present in the spore which contain the epitope recognised by the anti-EA1 antibody as a number of bands below the predicted molecular weight were observed. It has been suggested that this also occurs within the cell (Ariel *et al.*, 2003). Artefacts due to sonication seem unlikely, since profiles obtained for *B. anthracis*  $\gamma$ -irradiated Ames and UM23C12 strains using the same antibody showed significant differences. This suggests that different forms of the protein may exist within different strains, although whether this is significant in the spore is unclear. Furthermore, no detection of any 'contaminating material' was observed using FITC labelled anti-EA1 antibody. In contrast, the results seemed to suggest that antibody was binding predominantly to the spore. The reason why EA1 was observed associated predominantly with the spore may be due to the growth of the spores on a solid medium rather than in liquid culture. or variation in the epitope recognised by the antibody, the only two differing experimental factors compared to that reported previously (Williams and Turnbough, 2004). The purification of spores would not occur *in vivo* so it can not be discounted that selectively bound moieties may have as yet unknown functions within the infection process (Charlton *et al.*, 1999; Liu *et al.*, 2004).

The construction of a homology model of EA1 (Figure 5.9) suggested that EA1 is similar to an *E. coli* intimin (a C-type lectin, a family of  $\text{Ca}^{2+}$ -dependent lectins that show primary structural similarity in their carbohydrate-binding domains.) and a *Y. pseudotuberculosis* invasin. This supports the hypothesis that EA1 may have a role in the interaction with host cells and may have a role in the aerosolised route of infection

which has, to date, not been determined. This role may also suggest a reason for the inability to remove all EA1 from spore preparations. C-type lectins are found on the surfaces of a number of host molecules that have a role in the immune response and serve to bind to carbohydrate moieties exposed on the surface. Thus, it could be speculated that EA1 is bound to the carbohydrates moieties within the exosporium, of which a number have been described (Sylvestre *et al.*, 2002; Daubenspeck *et al.*, 2004; Waller *et al.*, 2005). Analysis of the surface proteins of an EA1 mutant, SM91, compared to UM23C12 suggested that the presence of EA1 masked other surface exposed moieties. Further experiments would be required to determine if antigen masking has any role in the pathogenicity of the *B. anthracis* spore.

One-dimensional (1D) and 2-dimensional electrophoresis (2DE) have been widely used for proteomic studies. When this is followed by a suitable technique for protein identification (commonly mass spectrometry based methods) this has shown promise for facilitating the identification of a large number of proteins from complex mixtures (Veith *et al.*, 2001; Krah *et al.*, 2003). Differences were seen in the protein profiles of *B. anthracis* compared to closely related species, particularly in the large number of lower molecular weight acidic proteins observed in *B. anthracis* this could account for the observed acidity of samples produced using this method and with exosporium preparations (C. Redmond personal communication). However in this study, 2DE was found to have limitations for the identification of proteins from spore preparations due to the high concentrations and volumes of spores that would have to be processed to produce sufficient protein for identification. Many of the problems with 2DE such as those relating to low abundance of proteins have been reported within other studies, prompting the use of alternative approaches (Molloy, 2000; Herbert *et al.*, 2001; Lilley *et al.*, 2002; Shaw and Riederer, 2003). The application of combinations of liquid phase based separation methods, in conjunction with mass spectrometry techniques have also proved an exceptionally promising alternative to 2DE (Wang and Hanash, 2003). Therefore, methods such as immunopurification and biotinylation of surface located components may prove invaluable in identification of proteins that may have important interactions in the infection process.

In this study, proteomics based approaches identified a number of novel proteins of the *B. anthracis* spore. Results show that the spore structure is complex and that the interactions of many of the individual components are unknown. The utilisation of



unique surface exposed regions of a protein could be invaluable in the production of recognition elements for specific identification where protein homologues are present in closely related species. This study has enabled the identification of four such targets that may be utilised for the specific detection of *B. anthracis* spores.

## **Chapter 6 Production of specific single chain antibodies against the *B. anthracis* protein EA1 for the detection of whole spores**

### **6.1 Introduction**

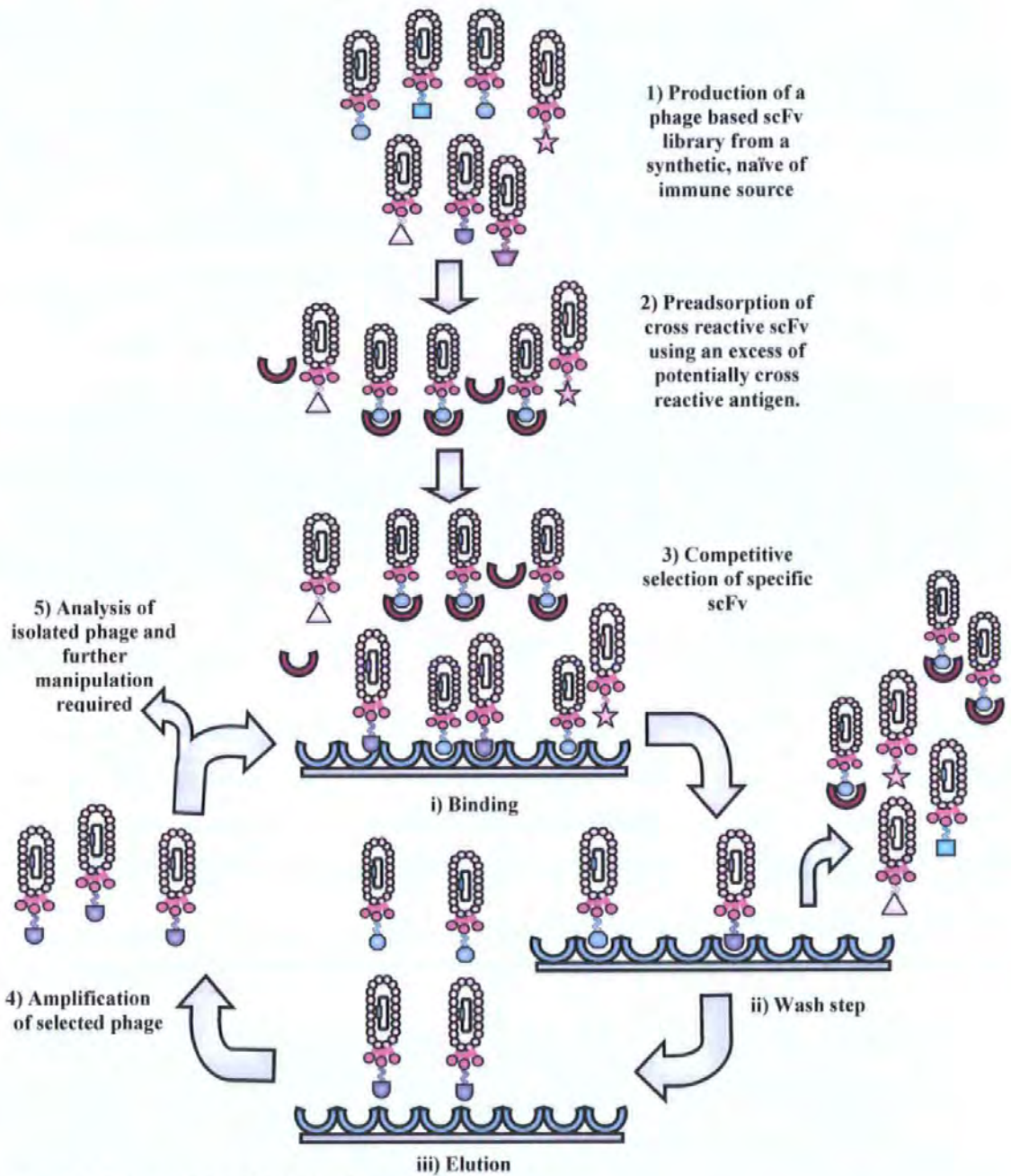
A preliminary step in the development of an immunoassay for the detection of a microorganism is the production of a highly specific antibody capable of binding to the live pathogen. Traditionally, poly (pAbs) or monoclonal antibodies (mAbs) produced against the whole organism have been used. In the case of polyclonal antibodies raised against whole cells or spores, the antibody will recognise a range of undefined antigens and epitopes. Furthermore, these epitopes may be present on other species, particularly if unique targets are rare or non-immunodominant. Monoclonal antibodies, although directed against a single epitope, may not be able to discriminate between highly conserved epitopes in related species. The use of detection reagents against unidentified and uncharacterised epitopes could cause significant limitations to the sensitivity and specificity of a detection assay as well as a reduction in the confidence of the results obtained.

In addition to the problems observed with lack of specificity and the recognition of unsuitable target proteins, because the production of antibodies is reliant on a mammalian immune response it is difficult to produce antibodies to toxic substances, or to unique, rare or non-immunodominant epitopes even though such targets could be suitable targets for detection assays (Emanuel *et al.* 2000). In the case of polyclonal antibodies, a specific target can be identified and antibodies produced through the use of affinity purified protein (Petrenko and Vodyanoy, 2003). However, this requires detailed knowledge of suitable targets and large quantities of purified target. Often this is achieved using recombinant protein, but this must also be expressed in the correct conformation. Additionally, such procedures are expensive and time consuming, particularly for large scale production.

Despite these limitations, production of natural antibodies has provided a platform that is the basis of many detection and diagnostic technologies, but this has often required the modification of detection technologies to optimise performance. For example, this may involve the use of additional reagents (Fratamico *et al.*, 1998), optimisation of assay conditions (Studentsov *et al.*, 2002), the modification of existing technologies or the development of new detection systems (Perkins and Squirrel, 2000).

Advances in recombinant DNA technology and computational molecular biology have allowed the production of alternatives to traditional antibodies that can be modified to suit the requirements of the detector and assay design (Irving *et al.*; 1996; Lindner *et al.*; 1997; Piervincenzi *et al.*; 1998; Jung *et al.*, 1999). Recombinant antibodies may be used to enhanced specificity to a chosen target. One of the distinct advantages of recombinant antibodies is that they can be designed or selected to discriminate between very similar proteins. This can be done either by experimental methods or by a process of rational design (Glaser *et al.*, 1992.; Cai and Garen 1995.; Kirkham *et al.*, 1999; Miyazaki *et al.*; 1999., McCarthy and Hill, 2001.; Skerra, 2000). Single chain antibodies (scFv), contain only the variable region, containing the antigen binding sites. The use of these molecules, which can be expressed in a range of vectors, such as bacteria, confers significant advantages over polyclonal and monoclonal antibodies as discussed in Chapter 2.

One method of producing antibodies against a given target is through the production of an epitope mimic (Hombach *et al.*, 1998). However, increased binding to the mimic compared to the native target has been observed (Goldbaum *et al.*, 1997). Other important techniques that have been reported are competitive panning and competitive elution. The use of a competitive panning procedure (sometimes termed pre-adsorption, subtractive antibody screening or negative selection) utilises pre and/or post adsorption steps to select out those scFv that bind to potentially cross reactive targets to produce specific scFv (Figure 6.1). This strategy is particularly significant in the production of antibodies to distinguish between closely related targets or to produce antibodies against two distinct epitopes. For example specific anti-melanoma antibodies have been prepared using a procedure of continuing the selection to the final round and then pre-absorbing with melanocytes 10 times (Cai and Garen, 1995). As an alternative to this method, Scherer *et al.* (1998) describe a methodology by which polyclonal antisera were produced against a specific target on human adipocyte cells. The strategy used was to deplete sera of antibodies that were not specific to the target by purification with the recombinant protein. The purified sera that recognised the antigen of interest was then used to construct a scFv library.



**Figure 6.1 Competitive Biopanning**

1) The scFv library is produced. 2) Potentially cross-reactive scFv are pre-adsorbed using target antigen for which cross reactivity is required to be eliminated. Commonly, this is added in excess of the antigen for which scFv need to be isolated. 3) During the selection phase potentially cross-reactive scFv are not available to bind to the antigen of interest and are ii) washed away and iii) the specific scFv eluted. 4) the phage are then amplified and further rounds of panning completed or 5) taken forward for further analysis.

An approach that would allow for the production of recognition elements that can discriminate between closely related species would be advantageous for sensitive and specific detection of *B. anthracis* spores. Zhou *et al* (2002) reported an approach to obtain antibodies to *B. anthracis* spores from a naïve human library, but cross reactivity with related species was observed. Williams *et al.* (2003) reported the development of peptide recognition elements to *B. anthracis* spores, but the target was unknown. Both of these approaches used whole spores where the detection target was not defined - the work described here uses a targeted approach to recognition elements production.

The use of a carefully selected target antigen that contains highly specific, non-conserved epitopes allows an increase in the specificity, sensitivity and confidence of a detection assay (Labadie and Desnier, 1992). Although a number of proteins were found to be components of the *B. anthracis* spore and released during sonication all of these proteins have homologues within the *B. cereus* group (Chapter 5). Furthermore, previous studies have not been successful in identifying a spore protein that is unique to *B. anthracis* (Ivanova., *et al.*, 2003, Todd *et al.*, 2003 and Redmond *et al.*, 2004). Therefore, completely unique targets may be rare and/or of low abundance, making identification of these proteins and production of antibodies by traditional methods difficult. In this study, examination of the protein sequence and the use of computer modelling allowed for the identification of four targets that contained unique regions, predicted to be exposed on the surface of the protein. Thus, these targets could potentially be used for the production of specific recognition elements.

The work described here addresses a targeted approach to the production of recognition elements. This was achieved through the production of single chain antibodies that specifically detected *B. anthracis* spores through recognition of an individual protein target namely the surface layer protein EA1. EA1 is a vegetative cell protein; however, this protein was consistently identified using a range of techniques described in Chapter 5 and is supported by previous studies (Lai *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004; Williams and Turnbough 2004). Although Williams and Turnbough (2004), suggest that this protein is a contaminant of *B. anthracis* spores and can be removed by Urografin purification and subsequent wash steps, this is in variance to the finding of this study (Chapter

5). EA1 is known not to be unique to *B. anthracis*, but also has homologues within other *Bacillus* species and the monoclonal antibodies tested were shown to cross-react with other *Bacillus* species. The aim of this work was to develop specific scFv to EA1. Once a specific recognition elements to a characterised protein has been produced demonstration of the detection of *B. anthracis* spores would be required. If the method was successful it could be applied to the other targets identified in Chapter 5.

## 6.2 Results

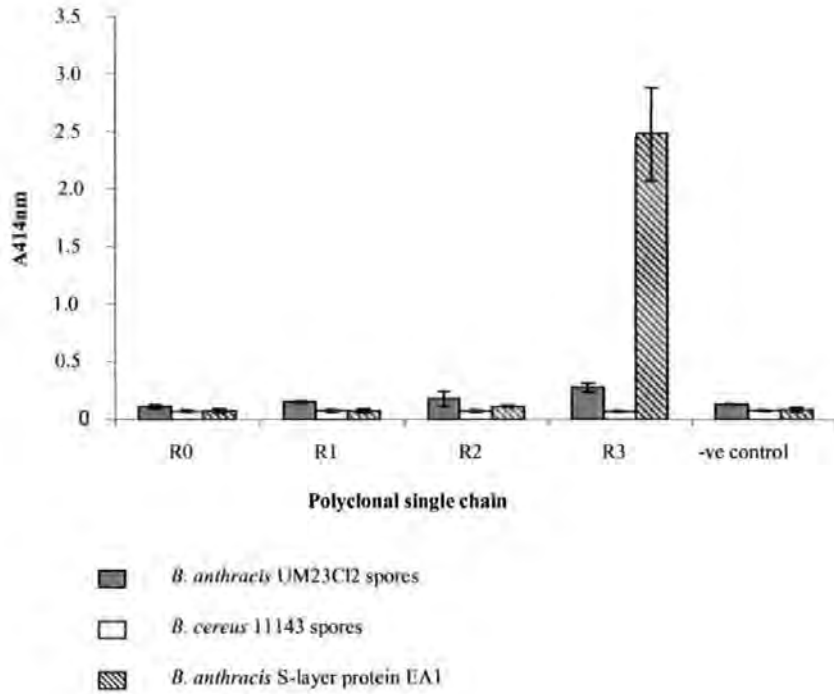
EA1 was prepared and purified (section 3.9). Confirmation that the purified protein was EA1 was undertaken by SDS PAGE, western blot using a monoclonal anti-EA1 antibody and mass spectrometry based methods (3.23.1.3-3.32.1.4).

### 6.2.1 Single chain antibodies produced by non-competitive panning

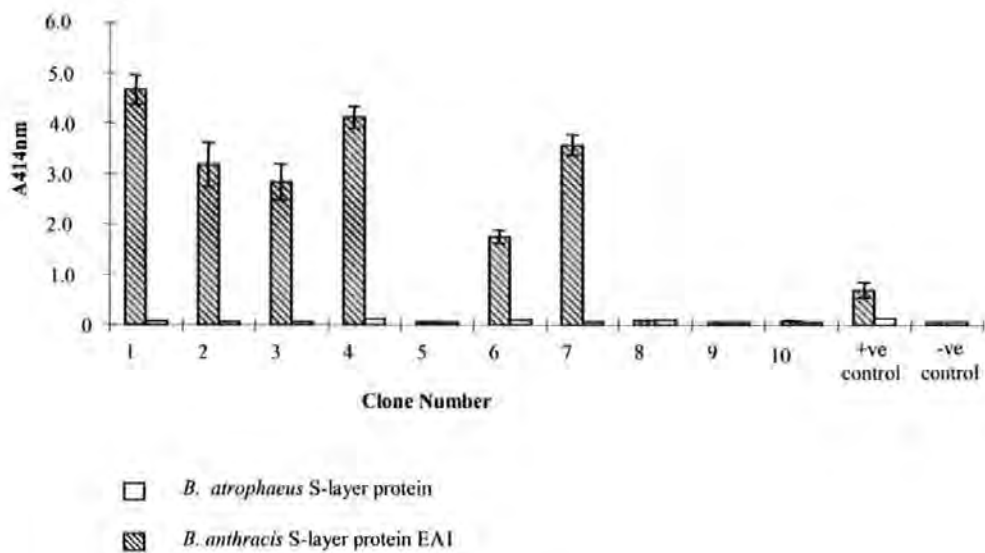
An immune library was constructed as detailed in 3.33 used the M13 bacteriophage as an expression vector as described by Krebber *et al.* (1997). The non-competitive panning strategy used here required three rounds of biopanning against the immobilised target antigen, purified EA1. The observed binding to EA1 in ELISA by the polyclonal population of scFv present after each round of selection showed an increasing signal after round 2 and 3 of selection (Figure 6.2). The immune library used was expected to contain antibodies to EA1 as the mice had been immunised with *B. anthracis* spores. This library would be expected to contain scFv to a range of spore and exosporium antigens, and has been used to produce antibodies to several other spore surface proteins in addition to EA1 (data not shown). The large increase in signal observed after round 3 is likely to be linked to the increased stringency of washing in this round (20 washes) compared with the lower stringency of the earlier rounds (10 washes).

After round 3 of non-competitive biopanning, 50 scFv clones were selected and their ability to bind EA1 assessed by direct ELISA. A sample result from ten of these clones is shown in Figure 6.3. Of the 50 scFv clones analysed 43 were found to bind to EA1. Only 2 of these 43 scFv clones demonstrated any cross-reactivity to purified *B. atrophaceus* S-layer protein. BstN1 fingerprinting of all the scFv clones that bound to EA1, demonstrated that there were

13 unique clones. These clones were all confirmed to be different by DNA sequencing and are referred to here as scFv1 to scFv 13.



**Figure 6.2** ELISA results showing binding of polyclonal anti-EA1 scFv to EA1 from each round of the non-competitive biopanning procedure. Polyclonal phage-displayed scFv from each round of the biopanning procedure (unpanned library designated round 0; R0-R3 represents the results of each panning round) were diluted 50% (v/v) in MPBS for detection of EA1. An anti-ovalbumin scFv was used as a negative control (labelled -ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate; error bars show two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.



**Figure 6.3** ELISA results demonstrating binding of ten monoclonal scFv to *B. anthracis* EA1 and to *B. atrophaeus* S-layer protein. Monoclonal phage-displayed scFv isolated from the third round of EA1 panning were prepared from 2 ml of supernatant by PEG precipitation and resuspended in 0.4 ml 2% (w/v) MPBS. An anti-ovalbumin scFv was used as a negative control (labelled -ve control), and a polyclonal scFv known to bind to *B. anthracis* spores were used as a positive control (labelled +ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate, error bars show  $\pm$  two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.

### 6.2.2 Sequencing data from scFv

The complementarity determining regions (CDRs) were defined using the Kabat definition as described by Martin (2001). Although a range of schemes have been described, the Kabat definition is used as a standard for the numbering of protein residues within the antibody sequence. Using these numerical data, the light and heavy chains CDRs of the antibody can be defined (Martin, 2001). Some sequences obtained from the DNA could not be analysed as, on translation, the sequence appeared to contain a large number of stop codons and are not



shown. In some instances the CDRs could not be determined as those sequences did not obey the Kabat definition (Martin, 2001).

The sequencing data revealed varying degrees of similarity between the CDRs for all the scFv analysed. In summary:

- (1) The light chain CDRs for scFv 1 were similar to scFv 2 except scFv 1 had 4 linker sequences and scFv 2 had 1.
- (2) The heavy and light chains of scFv 9 were similar to scFv 8, with only small deletions in CDR-L1 and CDR-L3.
- (3) The only sequence that was very different in all CDRs was scFv 6, which was found to cross react with a range of S-layer proteins.
- (4) In CDR-H1 a prominent consensus sequence was S Y G/D M S, which was also found to be a predominant consensus sequence from single chain antibodies produced against whole spores (C. Mayers personal communication.).
- (5) The highest degree of variability was observed within the protein sequences that form CDR-H3.

The CDR sequences are shown in tables 6.1 and 6.2.

The cross-reactivity of 13 unique scFv was determined by direct ELISA against S-layer proteins isolated from other *Bacillus* species. All of the 13 unique scFv tested showed cross reactivity with S-layer proteins from the other *Bacillus* species tested (for brevity, these ELISA results are summarised in Table 6.3). The highest degree of cross-reactivity was observed with *B. cereus* NCTC 11145 and *B. pumilus* S-layer proteins.

Antibody	Light chain CDRs																																	
	L1											L2								L3														
scFv 1	K	A	S	Q	N	V	G	T	N	V	A					S	A	S	Y	R	Y	S	Q	Q	Y	N	S	Y	P	L	M			
scFv 2	K	A	S	Q	N	V	G	T	N	V	A					S	A	S	Y	R	Y	T	Q	Q	Y	S	S	Y	P	W	T			
scFv 7	H	A	Q	N	I	N	V	W	L	S						K	A	P	N	L	H	T	Q	Q	G	Q	S	H	P	R	T			
scFv 11	R	S	S	Q	S	L	V	H	S	N	G	N	T	Y	L	H	K	V	S	N	F	R	S	S	Q	T	T	H	V	P	Y	T		
scFv 3	R	A	S	E	S	V	D	N	Y	G	I	S	F	M	N		A	A	S	N	Q	G	S	Q	Q	S	K	E	V	P	W	T		
scFv 6	S	A	S	S	S	V	S	S	S	Y	L	Y					S	T	S	N	L	A	S	Q	Q	R	R	S	S	P	P	T		
scFv 8	S	A	S	S	S	V	S	Y	M	H							S	T	S	N	L	A	S	Q	Q	R	S	S	Y	P	L	T		
scFv 10	S	A	S	S	S	V	S	Y	M	H							D	T	S	K	L	A	S	Q	Q	W	A	A	N	N	P	P	I	T
scFv 12	S	A	S	S	S	V	S	Y	M	H							D	T	S	K	L	A	S	F	Q	G	S	G	Y	P	L	T		
scFv 13	S	A	S	S	S	V	S	Y	M	H							D	T	S	K	L	A	S	Q	Q	W	S	S	N	P	P	I	T	
scFv 9	S	A	S	S	S	V	S	Y	M	H							S	T	S	N	L	A	S	R	S	R	Y	P	L	T				
scFv 5																	S	C	I	Q	P	R	I	Q	H	S	R	D	L	P	W	T		

**Table 6.1. The complementarity determining regions (CDRs) of the light chain of scFv produced by a non-competitive panning strategy**

Antibody	Heavy chain CDRs																																					
	H1									H2															H3													
scFv 1																																						
scFv 2	S	Y	G	M	S		T	I	S	A	S	G	T	Y	T	Y	Y	P	D	S	V																	
scFv 7	H	S	G	D	R	G	L	R	S	M	E	G	V	Q	G	Q																						
scFv 11																																						
scFv 3																																						
scFv 6	A	D	Y	Y	M	S	F	I	R	N	K	A	K	G	Y	T	T	D	Y	S	A	S	V	D	R	N	Y	G	S	H	W	Y	F	D	W			
scFv 8	S	Y	D	M	S		T	I	S	S	G	G	S	Y	T	Y	Y	P	D	S	V																	
scFv 10	S	Y	D	M	S		T	I	S	S	G	G	S	Y	T	Y	Y	P	D	S	V																	
scFv 12	S	Y	G	M	S		T	I	S	G	G	G	S	Y	T	Y	Y	P	D	S	V																	
scFv 13	S	Y	D	M	S		T	I	S	S	G	G	S	Y	T	Y	Y	P	D	S	V																	
scFv 9																																						
scFv 5	S	Y	G	M	S		T	I	S	T	S	G	T	Y	T	Y	Y	P	D	S	V																	

**Table 6.2. The complementarity determining regions (CDRs) of the heavy chain of scFv produced by a non-competitive panning strategy**

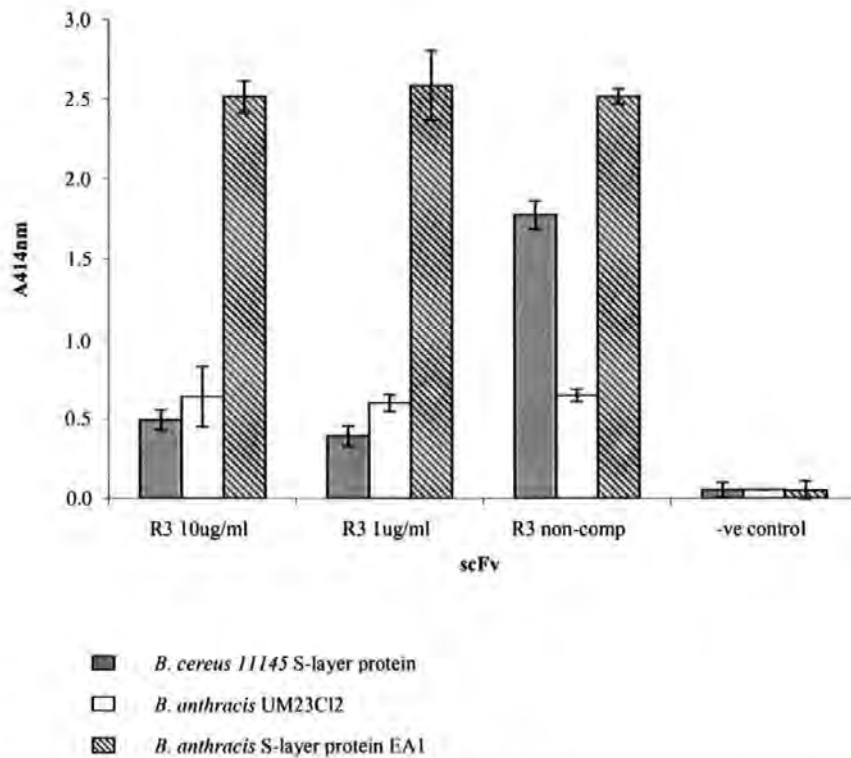
	<i>B. anthracis</i>	<i>B. thuringiensis</i> var. <i>israelensis</i>	<i>B. thuringiensis</i> var. <i>kurstaki</i>	<i>B. cereus</i> 11143	<i>B. cereus</i> 9946	<i>B. cereus</i> 11145	<i>B. pumilus</i>	<i>B. brevis</i>	<i>B. coagulans</i>	<i>B. anthracis</i> UM23CL2 spores
scFv 1	+++	-	-	-	++	-	-	-	-	++
scFv 2	+++	-	-	-	-	+++	+++	-	-	++
scFv 3	+++	-	-	-	-	+++	+++	-	-	++
scFv 4	+++	-	-	-	-	++	++	-	-	++
scFv 5	+++	-	-	-	-	+++	++	-	-	++
scFv 6	+++	-	++	++	++	+++	+++	++	-	++
scFv 7	+++	-	-	-	++	+++	++	-	-	++
scFv 8	+	-	-	-	-	+++	+++	-	-	++
scFv 9	+++	-	-	-	-	++	-	-	-	+
scFv 10	+	-	-	-	-	+++	+++	-	-	++
scFv 11	+	-	-	-	-	-	+++	-	-	++
scFv 12	+++	-	-	+++	-	-	-	-	-	++
scFv 13	+	-	-	++	-	+++	-	-	-	++
EA1.1	+++	-	-	-	-	-	-	-	-	++
EA1.23	+++	-	-	-	-	-	-	-	-	++
EA1.10	+++	-	-	-	-	-	-	-	-	+
EA1.20	+++	-	-	-	-	-	-	-	-	++
EA10.1	+++	-	-	-	-	-	-	-	-	++
EA10.4	+++	-	-	-	-	-	-	-	-	++

**Table 6.3. Summary of ELISA results demonstrating the binding of unique monoclonal scFv to EA1 and the cross-reactivity to other purified S-layer proteins, determined by ELISA.** Soluble scFv were produced, purified by immobilised metal affinity chromatography (IMAC) and used at 5 µg/ml in ELISA. Bound phage were detected using an anti-His tag HRP conjugated antibody (Sigma). Each assay was performed in triplicate. A summary of these ELISA assays is presented here; results are expressed in terms of the percentage of the maximum signal seen in the assay (+++ indicates 60-100% of the maximum, ++ indicates 20-59%, + indicates a signal greater than detection threshold, defined as the background signal plus 3 standard deviations from the mean of the background signal, and – indicates a signal below the detection threshold.)

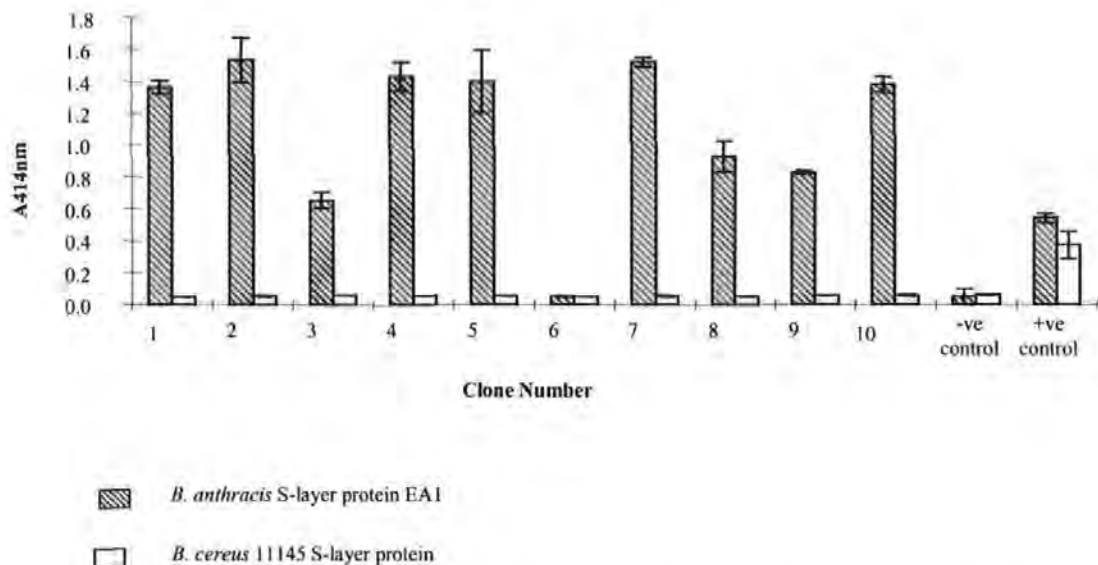
### 6.2.3 Single chain antibodies produced by competitive panning

After failing to isolate *B. anthracis* specific anti-EA1 scFv, a competitive panning strategy was employed. This involved negative selection of cross-reacting scFv by binding them to S-layer proteins from species that cross-reacted with the original anti-EA1 scFvs. The S-layer proteins were extracted from each *Bacillus* species and purified by gel-filtration chromatography (section 3.9). The panning procedure was repeated as detailed for the non-competitive strategy, but this time a mixture of competitive S-layer extracts (50 µg/ml *B. cereus* 11145 S-layer protein, and 25 µg/ml of *B. cereus* 11143, *B. cereus* 9946 and *B. pumilus* S-layer protein) were added to the solution of panning phage at the first panning round. The amount of EA1 used to coat the immunotube was also varied (1 or 10 µg/ml). Binding to EA1 in ELISA by the polyclonal population of scFv present after each round of panning showed a large increase in signal after the first round of panning. This could be due to the stringent negative selection in the first round that would have decreased the number of scFv that may bind to EA1. There was no significant difference in signal observed between the polyclonal scFvs selected using 1 or 10 µg/ml of EA1 (Figure 6.4). After round 3 there was still some cross-reactivity with *B. cereus* 11145 S-layer protein, however this was much lower than that observed in round 3 scFv phage using the non-competitive approach (Figure 6.4).

After round 3 of competitive panning, 50 scFv (25 from each of the 1 µg/ml and 10 µg/ml selections) clones were selected and analysed by ELISA. Ten of these are shown as an example in Figure 6.5. In total, 18 scFv cross-reacted with *B. cereus* 11145 S-layer protein (6 from the 1 µg/ml strategy and 12 from the 10 µg/ml strategy). Three of the selected scFv did not bind EA1, all of which were taken from the 1 µg/ml EA1 panning. 29 of the 50 scFv analysed were found to be specific for *B. anthracis* EA1; 16 of these were selected using 1 µg/ml EA1 and 13 were selected using EA1 at a concentration of 10 µg/ml. This result demonstrates the utility of the competitive panning method. It was not possible to obtain scFv antibodies specific to *B. anthracis* EA1 by conventional non-competitive panning, while the competitive method rapidly isolated non cross-reactive scFv antibodies that did not recognise *B. cereus* 11145 S-layer protein.



**Figure 6.4 ELISA results showing binding of polyclonal anti-EA1 from round 3 of competitive and non-competitive biopanning procedures.** Polyclonal phage-displayed scFv from round 3 of the biopanning procedure were diluted 50% (v/v) in MPBS for detection of *B. anthracis* EA1, *B. cereus* 11145 S-layer protein and *B. anthracis* UM23C12 spores. Three biopanning procedures were used; competitive panning with *B. anthracis* EA1 at 10  $\mu\text{g/ml}$  (labelled R3 10  $\mu\text{g/ml}$ ) or 1  $\mu\text{g/ml}$  (labelled R3 1  $\mu\text{g/ml}$ ), or non-competitive panning with *B. anthracis* EA1 at 10 $\mu\text{g/ml}$  (labelled R3 non-comp). An anti-ovalbumin scFv was used as a negative control (labelled -ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate, error bars show  $\pm$  two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.



**Figure 6.5** An example of ELISA results showing binding of anti-EA1 monoclonal scFvs selected by competitive biopanning procedures that show no cross reactivity with *B. cereus* 11145 S-layer protein. Monoclonal phage-displayed scFv isolated from the third round of EA1 competitive panning were prepared from 2 ml of supernatant by PEG precipitation and resuspended in 0.4 ml 2% (w/v) MPBS. An anti-ovalbumin scFv was used as a negative control (labelled -ve control), and a polyclonal scFv known to bind to *B. anthracis* spores were used as a positive control (labelled +ve control). Bound phage were detected using an anti-M13 HRP conjugated antibody. Assays were performed in triplicate, error bars show  $\pm$  two standard deviations from the mean. A positive result was defined as being higher than the average of the background signal plus three standard deviations of the mean background sample.

ScFv antibodies from rounds 1 and 3 of the competitive panning were examined by BstN1 fingerprinting to identify unique clones. As expected there was greater diversity of scFv after round 1 than after round 3 of panning. However, the diversity of scFv was much lower using the competitive strategy when compared with the scFv isolated by the non-competitive strategy. Only six unique scFv were identified in total from both the 1µg/ml and 10 µg/ml competitive panning strategies by BstN1 fingerprinting, and were confirmed unique by DNA sequence analysis (EA1.1, EA1.23, EA1.10, EA1.20, EA10.1, EA10.4). To distinguish the scFv from those using the non-competitive strategy the concentration of EA1 used and the clone number were used to identify the scFv. For example EA1.1 describes clone 1 from the 1µg/ml strategy. The CDR sequences are shown in Tables 6.4 and 6.5. The light and heavy chain CDRs for all scFv were similar except for EA10.1 which was dramatically different in all CDRs. EA1.1 was only different in one amino acid in CDR-L1 where arginine was substituted for alanine. EA1.23 also only differed in CDR-H3 where the sequence was extended by 9 amino acids. EA1.10 and EA10.4 were all exactly the same in all CDRs. When the entire antibody sequence was analysed there were minor differences in the surrounding scaffold sequence. EA1.20 differed to EA1.10 and EA1.10.4 by one threonine deletion in CDR-L1.





In CDR-H1 the consensus sequence was S F G M H whereas in the non-competitive scFv the predominant consensus sequence was S Y G/D M S. In a comparison of CDR-H2 for both the competitive and non-competitive scFv some differences are observed, as shown below

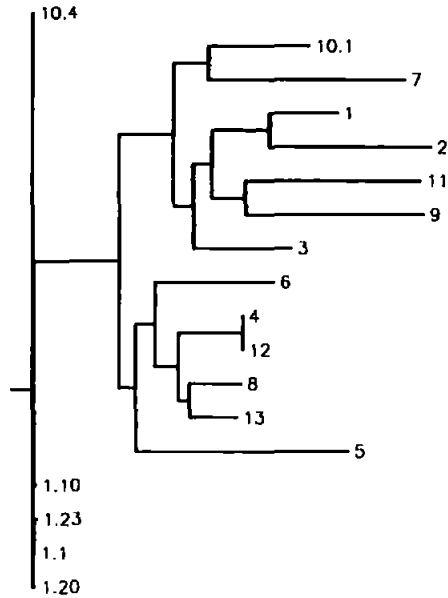
T I S A/T/G/S G T/S Y T Y Y P D S V (non-competitive)

Y I S S D G S T I Y Y A D T V (competitive)

In CDR-L3 the predominant sequence of the scFv from the competitive panning strategy was also found within scFv 5 from the scFv generated by the non-competitive method.

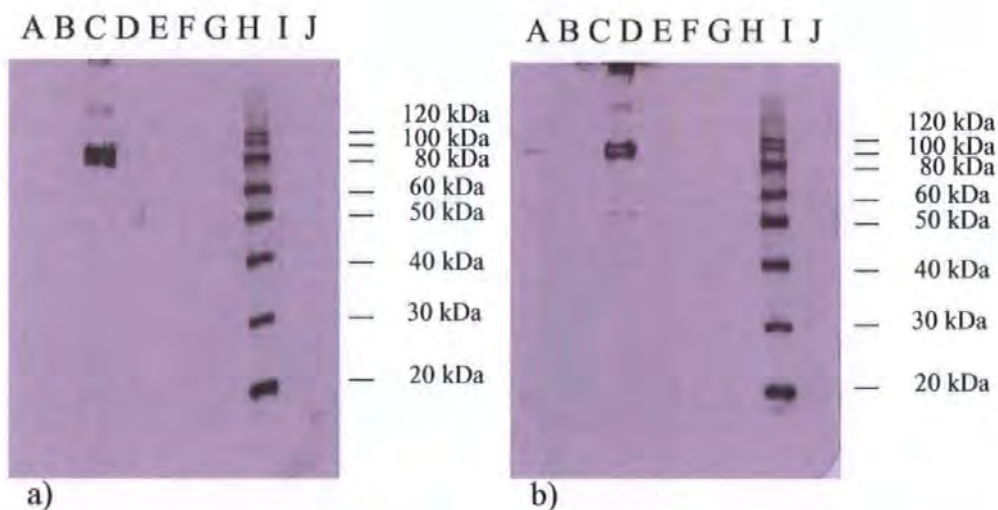
The only CDR that showed no similarities between the two methods used to produce  $\alpha$ -EA1 scFv was CDR-H3. In comparing the scaffold sequences for the scFv for both methods, similarities in the sequences were observed in the regions flanking the CDRs.

The similarities of whole sequences produced by both the competitive and non-competitive panning methods were ascertained using the CLUSTALW algorithm. This allowed the construction of a phylogenetic tree (Figure 6.6). Comparison of the sequences for the scFv obtained by the competitive and non-competitive panning strategies suggests that all those in the competitive group are very similar, with the exception of EA10.1 which shares higher identity with scFv 7 produced by the non-competitive method.



**Figure 6.6** Rooted tree diagram of the anti-EA1 scFv sequences obtained from non-competitive and competitive panning strategies. The tree was obtained using CLUSTALW.

The cross-reactivity of the six unique scFv isolated by competitive panning was determined by direct ELISA against S-layer proteins isolated from other *Bacillus* species. None of these scFv showed any cross-reactivity with S-layer proteins from the other *Bacillus* tested (Table 6.3). This indicated that all six scFv recognised epitopes are unique to *B. anthracis* EA1. To verify this result, the scFvs were used to probe purified *Bacillus* S-layer extracts on western blots. Detection of purified *B. anthracis* EA1 was demonstrated with all six scFv generated (two shown as examples in Figure 6.7). EA1.1, EA1.23, EA1.10 and EA10.1 showed no cross reactivity with any other *Bacillus* S-layer proteins tested, even in overexposed blots. EA1.20 and EA10.4 did show low levels of cross-reactivity with *B. pumilus* S-layer protein (only visible after a 30 minute exposure; example shown in Figure 6.7b). This cross-reactivity with *B. pumilus* S-layer protein was never observed by ELISA, Resonant Mirror or BIAcore analysis (data not shown).



**Figure 6.7 Cross-reactivity of monoclonal scFv selected by competitive biopanning to *Bacillus* S-layer proteins by Western blot.** The cross reactivity of two different antibodies is shown here; scFv EA1.1 (blot a) and scFv EA10.4 (blot b). Each S-layer protein extract was separated by SDS-PAGE(5  $\mu$ g per lane) on a 10% Bis-Tris NuPAGE gels (Invitrogen) and blotted onto nitrocellulose membranes (Invitrogen). Lane loading was as follows: A) *B. pumilus* S-layer protein, B) *B. brevis* S-layer protein, C) *B. coagulans* S-layer protein, D) *B. anthracis* S-layer protein EA1, E) *B. cereus* 11145 S-layer protein, F) *B. cereus* 11143 S-layer protein, G) *B. cereus* 9946 S-layer protein H) *B. atrophaeus* S-layer protein I) MagicMark™ Western standard, J) ovalbumin negative control. After probing, bound scFv was visualised using an anti-His tag HRP conjugated antibody (Sigma) followed by enhanced chemiluminescent detection.

#### 6.2.4 Use of specific EA1 antibodies on biosensors

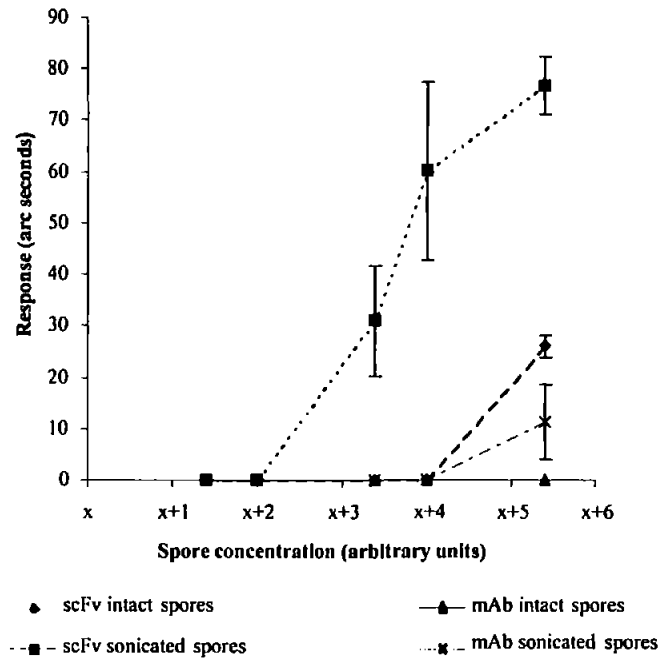
The S-layer protein EA1 was immobilised onto the surface of a BIAcore carboxymethylated dextran sensor surface (section 3.44). The scFv were passed over at concentrations of 5-400 nM (section 3.44). Analysis of the affinity constants of the six *B. anthracis* EA1-specific scFv on the BIAcore biosensor demonstrated that the highest affinity constants were seen in the scFv selected using 1 $\mu$ g/ml of EA1 compared to those isolated using 10  $\mu$ g/ml EA1 (Table 6.4). No binding was observed on the BIAcore with any of these six specific scFv for any S-layers evaluated (*B. pumilus*, *B. cereus* 11145, *B. cereus* 11143, *B. cereus* 9946, *B. coagulans*,

*B. brevis*, *B. atrophaeus*, *B. thuringiensis* var. *israelensis* and *B. thuringiensis* var. *kurstaki*; (data not shown).

Single chain	kA (1/M)	kD (M)
EA1.1	5.85E+10	1.71E-11
EA1.23	4.48E+10	2.23E-11
EA1.10	1.72E+08	5.81E-09
EA1.20	1.89E+10	5.28E-11
EA10.1	1.01E+09	9.91E-10
EA10.4	3.55E+08	2.81E-09

**Table 6.6. Equilibrium association (kA) and dissociation (kD) constants for scFv antibody clones produced using competitive panning strategies.** Equilibrium constants were derived using the BIAevaluation software (BIAcore) using a simple Langmuir 1:1 binding model and the association ( $k_a$ ) and dissociation ( $k_d$ ) rate constants calculated for each set of data. The equilibrium constant kA was calculated from the ratio  $k_a / k_d$  and kD from  $k_d / k_a$ .

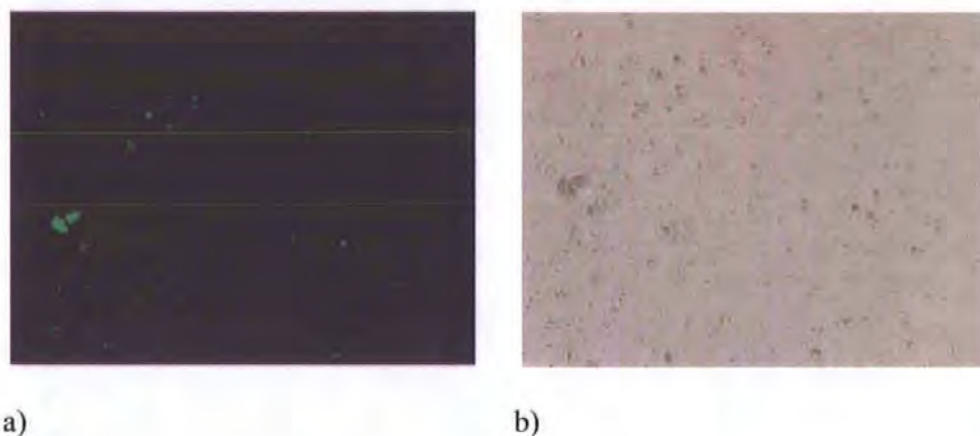
Demonstration of the detection of intact *B. anthracis* spores was carried out using the Resonant Mirror biosensor. Three of the specific scFvs (EA1.1, EA1.23 and EA1.10) were taken forward for evaluation of the sensitivity to whole *B. anthracis* UM23C12 spores on this real-time biosensor. For comparison, an anti-EA1 monoclonal antibody (mAb) was also evaluated. All scFv demonstrated detection of untreated *B. anthracis* spores (Figure 6.8), although the sensitivity of the assay improved significantly when the spores were sonicated. In comparison, the mAb could not detect intact *B. anthracis* UM23C12 spores at any of the concentrations tested, and only a small amount of binding was observed after sonication (Figure 6.8). The scFv showed no cross-reactivity to any other *Bacillus* species spores tested (both untreated or sonicated) while the mAb showed detection of sonicated *B. cereus* 11145 spores using this method.



**Figure 6.8** Detection of *B. anthracis* UM23C12 spores on a real-time biosensor using a competitively selected scFv (EA1.1) and a monoclonal anti-EA1 antibody. Antibodies were immobilised using standard EDC/NHS coupling onto a T70 CMD surface (Labsystems, Affinity sensors). Intact or sonicated spores were passed over immobilised scFv EA1.1 (scFv intact or sonicated spores) or anti-EA1 monoclonal (mAb intact or sonicated spores) antibody in PBS 0.05% (v/v) Tween 20. Spores were sonicated as described previously, using the tubular sonicator (Chapter 5). Error bars show  $\pm$  two standard deviations from the mean.

### 6.2.5 Recognition of Urografin purified spores using FITC-labelled scFv.

As was reported previously with the anti-EA1 antibody (Chapter 3), the results suggest that the scFv bound to whole spores that had been subsequently Urografin purified and washed according to the method described by Williams and Turnbough (2004). Representative samples are shown in Figure 6.9. No binding of FITC to the spore in the absence of antibody was observed, or binding of a non-specific FITC -labelled antibody was observed.

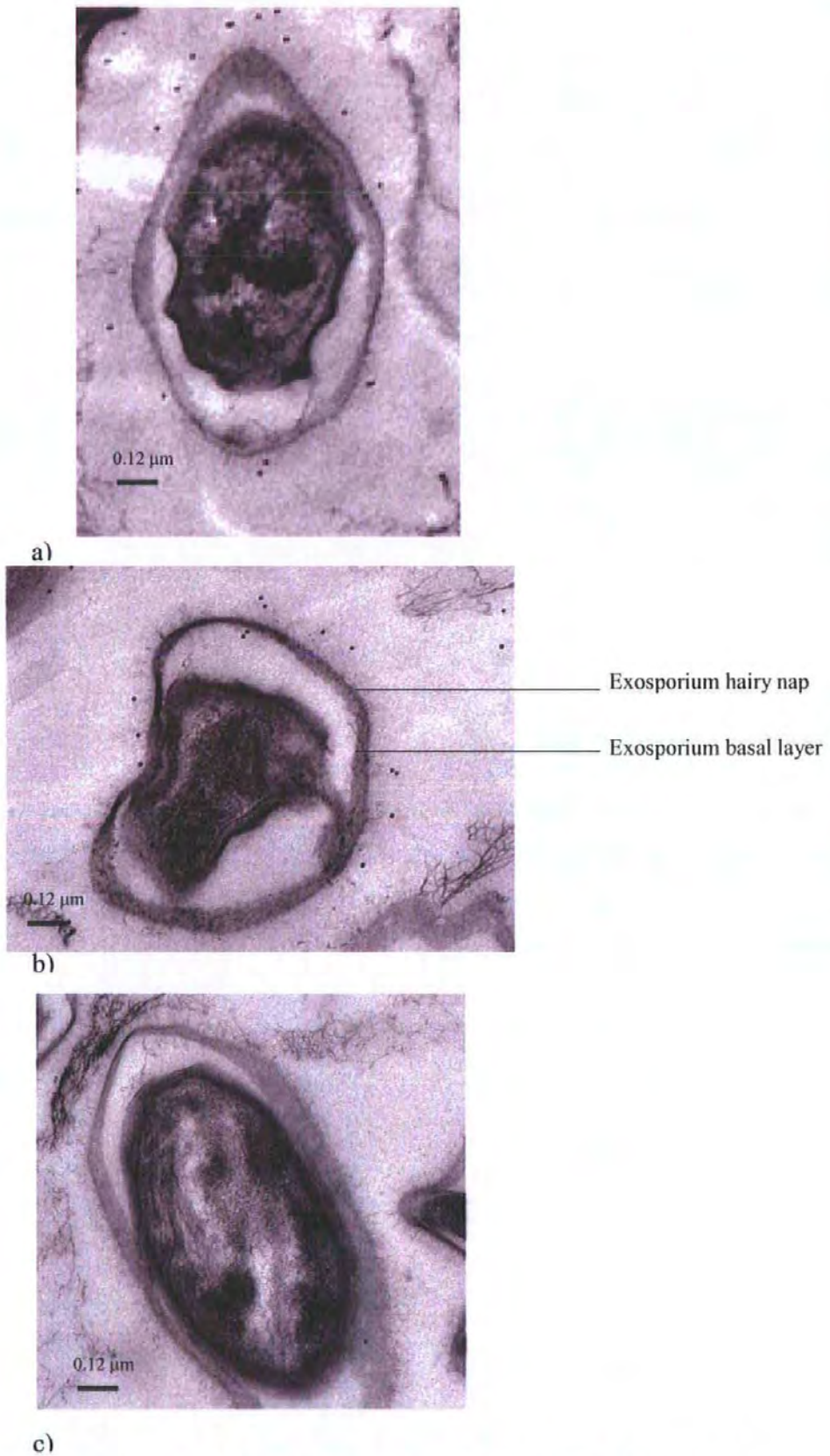


**Figure 6.9** Evaluation of the binding of a FITC labelled anti-EA1 scFv (EA1.1) to Urografin purified *B. anthracis* UM23C12 spores. a) *B. anthracis* spores washed 10x urografin purified and washed 3x viewed by fluorescence microscopy (x1000), b) *B. anthracis* spores washed 10x, urografin purified, and washed 3x, viewed by phase contrast microscopy (x1000).

### 6.2.6 Immunogold labelling

In order to complete the immunogold labelling experiments using the smallest number of conjugates the scFv DNA sequence has to be cut from the original plasmid and ligated into a pKAPPA plasmid, kindly provided by C. Mayers (Dstl) (section 3.47-3.48). This plasmid had the advantage of containing a murine kappa chain, so only a gold-labelled anti-murine antibody was required for immunogold labelling experiments (section 3.49). Immunogold labelling of thin sections of washed UM23C12 spores showed using the anti EA1 scFv, EA1.1 showed that most of the antibody was observed as being bound to the exosporium, more specifically the hair like projections within the nap of the exosporium. Some antibody was observed around the spore and not associated with the exosporium using this method. However, this was often observed within darker areas of the section, possibly indicating exosporium fragments. No binding of the gold label was observed in the absence of antibody. Representative sections are shown in Figure 6.10.

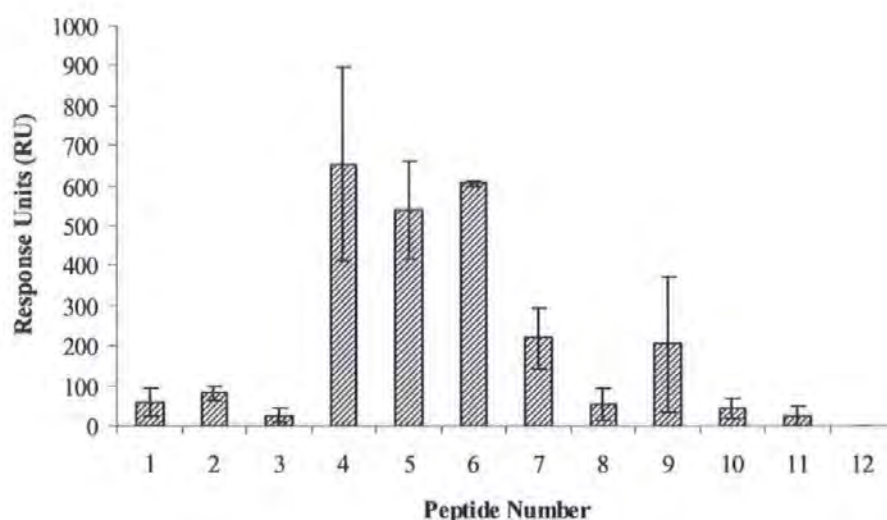




**Figure 6.10** Representative TEMs of immunogold labeled *B. anthracis* UM23C12 spores using scFv EA1.1 (a and b). A representative section from the negative control is shown in 6.10c.

### 6.2.7 Peptide mapping of linear epitopes

Linear peptides derived from the EA1 protein sequence (section 3.50) were synthesised using 14mers with a 4 aa overlap. Using the BIAcore all anti-EA1 scFv produced by the competitive panning strategy were immobilised onto a sensor surface and peptides passed over the surface at a concentration of to determine if they bound to the peptides (section 3.50). The results suggested that all of the antibodies bound in the same region, with the exception of EA10.4, to which no binding was observed to any linear epitope. Mapping of the linear epitopes to which a response was observed suggested that the epitope was contained within the region LNLPVDENAQPSFKDAKNIWI of the EA1 protein. An example of the results obtained is shown in Figure 6.11.



**Figure 6.11 Binding of linear peptides to immobilised scFv EA1.1.** Antibodies were immobilised using standard EDC/NHS coupling onto a T70 CMD surface (Labsystems, Affinity sensors). Peptides were passed over for 10 min. Error bars show  $\pm$  two standard deviations from the mean.



### 6.3 Discussion

The aim of producing the scFv was for use in an optical biosensor, in order to demonstrate the near real-time detection *B. anthracis* with the exclusion of all other *Bacillus* species. Therefore, demonstration that the anti-EA1 scFvs would not react with S-layer proteins from other *Bacillus* species was required. It is often argued that any antibody can cross react with another unrelated target, leading to the observation that “an antibody is only truly specific in the background in which it is tested” (Holt *et al.*, 2000). In a biodetection context, the relevant background is the background that the sample would have been isolated from, usually an environmental air sample. For this reason only a selection of *Bacillus* species was tested for cross reactivity. An organism of particular concern was *B. thuringiensis*, used (and sprayed) widely as an insecticide. BLAST searches demonstrate a very high degree of similarity between the EA1 protein sequence of *B. thuringiensis* and *B. anthracis*. The other organism of concern was *B. cereus*, another closely related species found widely in soil (Helgason *et al.*, 2000). The high cross-reactivity of the scFv produced by non-competitive biopanning suggests that the S-layer proteins of these species may contain proteins that are homologous to the *B. anthracis* EA1 S-layer protein. The higher degree of cross-reactivity observed with spores of *B. pumilus*, *B. atrophaeus* and *B. cereus* 11145 suggests that these proteins demonstrate the greatest degree of similarity to EA1, or that a highly conserved or immunodominant epitope exists within these species (Holt *et al.*, 2000). Irrespective of the reason why these scFv were cross-reactive, none where of any use for specific detection of *B. anthracis*.

The ELISA results from the competitive biopanning strategy indicate that some scFvs that cross-react with *B. cereus* 11145 S-layer protein were still selected, even though a competitive selection procedure was used. This may be because the cross-reactive antigens from closely related species were not sufficiently in excess (although 10-fold excess was used) leaving cross-reactive scFv to bind to the target. Furthermore, the cross reactive epitope within the S-layer protein that binds to the scFv may be immunodominant, ensuring that a large proportion of the scFvs bind to this site (Holt *et al.*, 2001). It may also be due to selective pressures imposed by growth or expression, or a combination of these factors. Despite the cross reactivity of the round 3 polyclonal scFv population it was hoped that some scFv clones with reduced cross-reactivity would have been selected.

Results of the sequence analysis after competitive panning suggests that this process rapidly led to the elimination of a large proportion of the population of scFv clones by negative selection. This could suggest that some binders were lost through further rounds of selection, either due to low affinity or selection pressures of bacterial growth or expression. The scFvs produced by the competitive approach showed less diversity in the CDRs than those produced by competitive selection. As expected, variations in CDR-H3 gave the main source of diversity between the different antibodies, as CDR-H3 is mainly responsible for specificity (McCarthy and Hill, 2001)

The results for the determination of affinity constants ( $K_A$  and  $K_D$ ) revealed that these *B. anthracis* specific scFvs had apparent nanomolar affinities for EA1, suggesting sufficient affinity for them to be used for sensitive detection. As absolute specificity was used as the original criterion for success, other stronger binders (albeit cross-reactive) may have been eliminated through the competitive panning process. It is likely that the affinities of these antibodies could be improved by further maturation techniques if desired (for example, Jermatus *et al.*, 2001; Mossner and Pluckthun, 2001).

The results of the epitope mapping suggest that all the scFv, with the exception of EA10.4 bind the same epitope, this supports sequence data for the scFv. This is perhaps unsurprising as EA10.4 showed the least similarity in sequence compared to the other scFv. The epitope to which EA10.4 bound could not be determined using this approach so it is likely that this antibody binds to a discontinuous three dimensional epitope. Interestingly, the areas to which the scFv bound correlate in part to the unique sequences identified as being surface located (Chapter 5) by BLAST, as shown in Figure 6.12. In comparison to the predicted unique regions highlighted in Chapter 5 (Figure 5.10-5.11), one of these regions DAKNIWS, was also predicted to be located on the surface of the protein. However, the other area to which the antibody bound, PVDENA, only showed one amino acid substitution compared to closely related species such as *B. cereus* E33L. This region was also not predicted to be surface located. These amino acids (PVDENA) may have no role in determining the specificity of a recognition elements, alternatively it may be an important residue for the structure of the surface located region, or the modelled structure may be incorrect and this region may also be surface located. These results suggests that a assumption of binding to linear exposed regions, whilst possible, may be over simplified; however, this approach is at least suitable for identifying the potential to produce specific recognition elements. In future work smaller

peptide sequences could be used to define the epitope to which the antibody bound more clearly and amino acid substitution could be used to determine those residues which are essential for antibody binding.

<i>B. anthracis</i>	LNLPVDE	NAQPSFKDA	KNIWSSKY	24
	LNLPVD	NAQPSFKDA	KN W++KY	
<i>B. cereus</i> E33L	LNLPVDD	NAQPSFKDA	KNTWAAKY	106

**Figure 6.12** Areas of difference in the EA1 sequence in the regions bound by selected scFv. Difference between *B. anthracis* and *B. cereus* E33L S-layer protein within the region are highlighted in red.

Usually, scFv libraries that are produced from immunised mice are used to obtain scFvs that show high affinities for the desired targets. While naive libraries have been used successfully, it has been documented that higher affinities and a wider diversity of antibodies can be obtained from an immune library. Competitive panning is extremely useful in this case to reduce the complexity of these immune libraries by eliminating cross-reactive antibodies. Immune libraries have the advantage of having undergone significant affinity maturation in the mouse; the antibodies evolve *in vivo* in the B cell by hypersomatic mutation within the hypervariable regions to enhance specificity and affinity (Borrebaeck and Ohlin, 2002).

The immune library used here was created from mice immunised with a complex antigen (whole *B. anthracis* spores) not specifically against EA1, so this library would have contained a selection of antibodies that bound to a range of target antigens. The diversity of this library may be limited with respect to those that recognise a range of EA1 epitopes. If an  $\alpha$ -EA1 library had been utilised, this may have enhanced the probability of isolating an EA1 specific scFv by non-competitive selection, although in practice it is preferable to producing a single library for each complex target, allowing a range of antibodies to be isolated whilst minimising animal use. It is possible that this method may only be applicable to highly immunogenic antigens such as EA1. Western blot analysis of a polyclonal goat antiserum raised against whole *B. anthracis* spores also showed binding to EA1, demonstrating that it is present and immunogenic in spore preparations (data not shown). It is important to remember that the specific EA1 antibodies must have been lost during non-competitive panning, perhaps

due to competition from non-specific antibodies with higher affinities for the target or other selection pressures such as slow growth.

Unlike previous applications of competitive panning (for example, Cai and Garen, 1995; Scherer *et al.*, 1998; Krebs *et al.*, 2001), the much simpler procedure of one step negative selection demonstrated here shows that a complex mixture of competitors can be used in the first round of selection to remove the majority of non-specific binders and isolate a number of scFvs specific for the original target.

As previously discussed (Chapter 5) it has been suggested that EA1 is a contaminant within spore preparations and could be removed through the use of Urografin purification (Williams and Turnbough, 2004). However, this does not agree with the observations made in this work. After labelling the scFv created with FITC and allowing the antibodies to bind to Urografin purified and washed spores the antibodies were found to bind to whole spores and not any contaminating material within the sample. This confirms previous observations using an  $\alpha$ -EA1 mAb (Chapter 5).

The insertion of the scFv sequence into the pKAPPA construct was advantageous for immunogold labelling as this construct had been designed to incorporate a His-tag for purification but also murine kappa chain. Therefore, the gold particle would be kept as close to the site of binding as possible as only an anti-mouse gold conjugate was used. If the scFv had been used in the previous constructs, containing only a His-tag, a series of antibodies would have to have been used to incorporate an anti-species gold conjugate. Immunogold labelling of whole spores revealed that most of the antibody was bound to the hairy nap of the exosporium. Although selective binding of EA1 to the exosporium may be fortuitous, a function for EA1 within the spore (in particular *in vivo*) can not be discounted until further experiments are conducted (Charlton *et al.*, 1999; Liu *et al.*, 2004). The fact that the antibody bound to the hairy nap suggests that EA1, or the EA1 epitope recognised by the antibody, is present within this layer. A small proportion of antibody was visualised surrounding the spore; on closer examination this was observed associated with darker material within the section, possibly

fragments of the exosporium. However, these hypotheses would have to be assessed in future work. The results to date do however, suggest that EA1, either whole or in a truncated form, has a stronger association with the exosporium than previously suggested by Williams and Turnbough (2004).

The generation of specific recognition elements for EA1 and consequently *B. anthracis* spores in this case, was only possible using the competitive strategy. The resultant recombinant antibodies should be useful across a wide range of detection techniques, from the conventional laboratory analysis methods such as ELISA and Western blotting to detection technologies capable of near real time detection such as evanescent wave based biosensors. The use of these specific recognition elements on the Resonant Mirror biosensor enabled the detection of anthrax spores in near real time, with no false positives even when exposed to very high backgrounds of closely related *Bacillus* species that commonly cross-react with anti-anthrax polyclonal and monoclonal antibodies. This is the first demonstration of antibodies that are able to perform to this high specification have been described.

## **Chapter 7 General Discussion and Conclusions**

In this study, three main areas were addressed in order to achieve the near real time detection of *B. anthracis* spores. The first issue was to address the theoretical limitations associated with the detection of larger particulates using optical biosensors (Watts *et al.*, 1994; Framatico *et al.*, 1998), by disrupting the spore prior to the assay. Secondly, it was necessary to characterise the antigenic and other components of the spore that were surface accessible and/or released by the disruption step. Finally a specific ligand was developed for detection.

A preliminary survey of disruption techniques, and the requirement for a rapid flow through system that could disrupt the spores prior to the detection assay, suggested that electroporation and sonication were the two most promising approaches. Although these two techniques have been utilised for many years for various purposes, they still remain relatively uncharacterised, particularly in the case of bacterial spores. Furthermore, the use of such techniques for enhancement of immunoassay-based techniques has not been described.

It was found that electroporation did have an effect on bacterial spores, observed as sub-lethal alterations of the outer integuments and the release of components into the surrounding medium; this is an observation that has previously been widely dismissed with the exception of one study by Marquez *et al.* (1997). However, the mechanisms by which this occurred could still only be speculated upon. Further experiments are required to determine any alteration to the underlying structures of the spore and to fully understand the mechanisms by which this occurs. Using the conditions tested, the electroporation of spores did not confer an increase in assay sensitivity probably due to the denaturation of protein and/or migration and deposition of proteins and DNA on the electrodes.

In the case of sonication, preliminary work suggested that heat was a limiting factor, as a number of cooling steps were required to allow for recognition of the proteins released. The development of a radial transducer allowed for in line disruption of the spores whilst limiting heating of the bulk solution. Using this system, significant improvements in the sensitivity of the detection of *B. anthracis* spores (up to 500 fold) was demonstrated, using an optical biosensor and other immunoassay based techniques. Therefore, the application

of disruption to enhance the sensitivity of the near real time detection has been shown experimentally for the first time and could have significant implications for a range of immunoassay based technique for detection and diagnosis.

As in previous studies, defining the effect of ultrasonic cavitation was difficult. The reasons for this are probably two-fold, firstly due to the complexity of the numerous possible outcomes of sonication such as high local heating, shear forces, and the production of free radicals and secondly due to the difficulty in characterising and predicting of the outcomes of these effects on a complex moiety such as a spore. The use of the tubular sonicator, where bulk heating was maintained at definable levels, confirmed that the predominant effect of sonication on the bacterial endospore was to remove the outer integuments of the spore, probably due to cavitation events and microstreaming within the surrounding media. Previous observations of internal damage were probably largely attributable to heat, as they were not observed using this system. Although preliminary results suggest that ultrasonic cavitation may have an effect on the proteins released, as has been suggested by Strathopoulos *et al* (2004), this appeared to be negligible for antibody based detection using the conditions tested. The increase in assay sensitivity was probably due to the release of proteins from the surface resulting in an apparent increase in analyte concentration, increased mass transport to the sensor surface and a reduction in steric hindrance. Therefore the choice of antibody antigen pair would be essential in achieving the optimal assay sensitivity. For antibody based detection the immunogenicity of a target would also be advantageous. To this aim, the structural and surface located antigenic components of the *B. anthracis* spore were determined.

The exosporium of *Bacillus anthracis* represents the primary barrier between the spore and the external environment and as such represents a source of putative detection targets. The identification of the immunodominant exosporium protein BclA has already been reported; however, this glycoprotein shares high identity with homologues of closely related species which have been identified experimentally in *B. cereus* and *B. thuringiensis* spores and as such is not suitable for specific detection (Sylvestre *et al.*, 2002; Steichen *et al.*, 2003). In this study, the identification of other immunogenic proteins of the *B. anthracis* exosporium was achieved through the development of an immunopurification procedure. Using this technique, 9 proteins were identified, 8 of which have been described previously in exosporium preparations (Steichen *et al.*, 2003; Todd *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004). A second CotB homologue was also identified experimentally as a

component of unwashed *B. anthracis* exosporium, which has not been reported previously. The nature of the association of alanine racemase with the exosporium is still unclear, although preliminary results suggest this enzyme may interact with other immunogenic proteins, ExsY, ExsK and CotY. ExsFA (ExsF/ BxpB) was also identified as an immunogenic protein and has also been widely cited as a component of the exosporium from both *B. anthracis* and *B. cereus* spores (Steichen *et al.*, 2003; Todd *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004; Sylvestre *et al.*, 2005) and has been reported to be associated with BclA (Steichen *et al.*, 2003, 2005; Redmond *et al.*; 2004; Sylvestre *et al.*, 2005). This is the main structural component of the fine filaments of the exosporium hairy nap. Along with ExsFA, a second ExsF protein, ExsFB appears to have an important structural role within the exosporium. Together, these ExsF proteins may be required for the proper localisation of BclA to the spore surface and for the stability of the exosporium crystalline basal layers (Sylvestre *et al.*, 2005). The ExsF proteins are probably organised in multimeric complexes with other exosporium proteins (Redmond *et al.*, 2004; Sylvestre *et al.*, 2005). The immunogenicity of ExsF (ExsFA) was also shown in a recent study by Sylvestre *et al.* (2005).

The identification of immunogenic proteins of *B. anthracis* may suggest a more primary role in anthrax infection as well as being important in the development of immunoassays for detection and diagnostic purposes. The exact function of the majority of the proteins identified in this study and their location within the outer spore surface layers has yet to be elucidated. However, these results and other work suggest that complex interactions exist between identified exosporium proteins in the formation and assembly of the spore coat and exosporium (Bailey-Smith *et al.*, 2005; Sylvestre *et al.*, 2005). Future experiments will seek not only to answer these questions, but the methodology developed using polyclonal antibodies from different species and antibodies raised against different micro-organisms could be utilised to identify immunogenic proteins. This technique could possibly provide an important step in the identification of antigenic markers for the development of detection and diagnostic assays and medical countermeasures including vaccines, as well as enhancing the understanding of markers that may be important in pathogenesis.

A number of proteins were identified following sonication of *B. anthracis* spores using the tubular sonicator, some of which have not been described previously as spore proteins from experimental observations. The results suggested that this system could be used to increase the number of putative detection targets to beyond those located in the exosporium. In the absence of proteins specific to *B. anthracis*, computer modelling



techniques were used to evaluate the location of unique regions within the protein that were predicted to be surface accessible. This allowed for rational selection criteria to be implemented to select targets for which specific ligands could theoretically be produced. To date EA1, BA3189 (MntA), BxpA and ExsA may function as such targets. BxpA has been described as a component of the *B. anthracis* exosporium (Steichen *et al.*, 2003). However, MntA and ExsA represent novel *B. anthracis* spore proteins.

MntA (BA3189) has been found to be a chromosomally encoded virulence factor of *B. anthracis* (Gat *et al.*, 2005). Although reported previously as being present predominantly in the cell (Gat *et al.*, 2005), in this study it was also isolated from spore preparations. In the work reported by Gat *et al.* (2005) a *mntA* deletion mutant was found to demonstrate retarded growth in rich media, delayed release from cultured macrophages and increased sensitivity to oxidative stress. A role in increased oxidative stress is of interest, as work conducted by Kang *et al.* (2005) suggests that a role of the exosporium may have a protective role within the host macrophage. Thus, proteins such as superoxide dismutases, alkyl hydroperoxide reductases (such as BA0345 identified in this study) and other identified components of the outer spore layers (Lai *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004; Steichen *et al.*, 2005) may also have a role in this function. Future experiments will seek to address this, for example by the use spores in which of the genes which encode these proteins have been deleted and subsequent evaluation of survival within the macrophage.

The ExsA protein has not been reported previously within the *B. anthracis* spore. Work conducted by Bailey-Smith *et al.* (2005) showed that the ExsA protein of *B. cereus* (ATCC 10876) has a functional role in the formation of the spore coat and exosporial layer. The ExsA proteins are homologues of SafA of *B. subtilis*. The SafA protein has been found to be associated and directly interact with SpoVID during the preliminary stages of the assembly of the spore coat and is located at the interface between this layer and the spore coat in mature spores (Ozin *et al.*, 2000, 2001). Localisation of SafA within the spore is also dependent on SpoVIA (Ozin *et al.*, 2001). As with *B. cereus* (Bailey-Smith *et al.*, 2005), SpoVID and SpoVIA also have homologues within the *B. anthracis* genome and thus may be required for the assembly of ExsA (Bailey-Smith *et al.*, 2005). The requirement for ExsA for the assembly of the coat and exosporium suggests that this protein may have an essential function and thus would make a suitable target for detection and diagnostics assays. In this study, the sonication of *B. anthracis* spores only appeared to

disrupt the exosporium and to a lesser extent the underlying spore coat. Thus, since ExsA is required for formation of the exosporium taken in combination with the known location of SafA, it could be speculated that ExsA may also be located at the interface between the spore coat and exosporium. Further experimentation using ligands, such as scFv, generated against the ExsA proteins and subsequent immunogold could be used to determine if this hypothesis is correct.

The direct identification of proteins specific to the *B. cereus* group through direct analysis of pepsin digests obtained from sonicated spores may have significant implications for mass spectrometry based identification. In previous work, Small acid soluble proteins (SASPs) have been extracted following exposure to acid and identified by matrix assisted laser desorption ionisation mass spectrometry (MALDI-MS) (Elhanny *et al.*, 2001; Hathout *et al.*, 2003; Warscheid *et al.*, 2003; Warscheid and Fenselau, 2003; Horneffer *et al.*, 2004; Whiteaker *et al.*, 2004; Pribil *et al.*, 2005). Generic identification of the presence of spores is certainly possible using these methods; however, discrimination at the species level is more problematic as this is often attributable to a single tryptic digest. More recently presented work has questioned the reproducibility of the identification of the key peptides required for discrimination at the species level (Jue *et al.*, 2005). Thus, although SASPs may be useful for mass spectrometric identification they may not always provide a reliable method of distinguishing different species. The results obtained here using a physical disruption step and subsequent MS/ MS identification may provide more reliable markers for species specific identification. However, the statistically significant reproducible identification by MS would need to be assessed.

In variance with previous studies (Williams and Turnbough, 2004), EA1 was identified in Urografin purified spores and was consistently identified using a range of techniques. This protein has been previously identified in spore coat extracts and exosporium (Lai *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004). Preliminary evaluation of monoclonal antibodies and scFv that recognised EA1 were found to bind to the spore and not to any contaminating material within the spore preparation. Immunogold labelling confirmed these observations and determined that a significant proportion of antibody was bound to exosporium, more specifically to the hair-like projections of the nap. This observation, combined with data obtained from homology models suggested that EA1 was located within the hairy nap, possibly bound to the carbohydrate component of BclA and/or BclB. One potential problem with the use of numerous washing and purification steps is that the fragility of the exosporium to these conditions has not been determined. Therefore, the

release of exospore fragments during such processes can not be discounted (Pembrey *et al.*, 1999). Although not proven, this could be considered a possible reason for the decrease in the amount of EA1. Ideally, quantification of known structural components before and after treatment would be needed to assess the effect of the purification procedures. Further work would then be required to determine if the purification process had any effect on pathogenicity due to the possible loss of exosporium components. Although EA1 may be a contaminant of these preparations, the selective adsorption to the exosporium, as with other non-structural components, may have an as yet undescribed function and as such EA1 was not disregarded as a target for specific detection. As the role of EA1 within the spore is unclear, but since it had been consistently identified from spore preparations and was not unique to *B. anthracis* this target was taken forward for the production of specific ligands.

Monoclonal anti-EA1 antibodies were not specific to *B. anthracis* and were found to cross-react with closely related species. The generation of specific recognition elements for EA1, and consequently *B. anthracis* spores, was possible through use of a competitive biopanning strategy. The use of the resultant  $\alpha$ -EA1 scFv demonstrated real-time detection of *B. anthracis* spores. Research to date using pre-adsorption has not demonstrated the elimination of cross-reactivity to such a broad range of cross-reactive targets in one single step. Therefore, although EA1 may not represent the 'ideal' detection target, the methodology used could be implemented for other proteins of the *B. anthracis* spore. In conclusion, the method of competitive panning described here provides a significant advantage over conventional monoclonal and polyclonal approaches, particularly for critical diagnostic and detection applications.

Proteomics is a rapidly expanding area and as more sequence data from various organisms becomes available, the ability to select detection targets for the production of detection ligands is increasing. The approaches described within this study could be useful in the identification of novel proteins to which specific ligand could be generated. Conversely, as sequence data is added the number of unique regions may decrease, as was found during this work, thus creating an impetus to define the biological background from which the sample may be derived, as well as knowledge of the presence and abundance of proteins within closely related bacteria.

The use of BLAST searches and computer modelling were useful in the selection of potential targets for the production of specific ligands; however, the presence of a gene

does not indicate whether it is transcribed. Furthermore, examination of the genomes of closely related species suggests that the evolution of a pathogenic species can occur through the expression and regulation of genes as well as the acquisition of additional genes. Thus, one important consideration would be the determination of the presence of proteins within closely related species. This in itself presents a problem because although large scale analysis of the proteins present in closely related species could be achieved by microarray analysis, this technique has inherent problems and would be limited to genes induced in sporulation as described by Liu *et al.* (2002) and the number of organisms required to be analysed by this method would take considerable time. The alternatives are the use of techniques such as Isotope Coded Affinity Tag (ICAT) and the Absolute Quantification of proteins and post translational modification (AQUA) that can quantitatively determine the concentration of a protein of interest, without the need for an antibody (Gerber *et al.*, 2003). Again, this procedure would be relatively time consuming for the number of organisms to be evaluated and is limited to proteins suitable for such analyses. Computer modelling techniques are confined by ability to obtain accurate sequence and structural information for the proteins of interest.

The identification of a target that fulfils all the criteria of surface location, specificity, abundance, required for virulence and expression under all possible conditions seems unlikely considering this and other research. For example, using mass spectrometry based techniques, CotY and ExsY have been shown to be two of the most abundant proteins within exosporium preparations (Liu *et al.*, 2004). Examination of genomic data (Todd *et al.*, 2003) has shown that in *B. cereus* and *B. anthracis* the *exsF*, *exsY*, and *cotY* genes are located in a gene cluster close to other genes thought to be required for formation of the exosporium. Results from this study also indicate that these proteins are immunogenic. However, these proteins show high degrees of identity with closely related species as such it is unlikely that specific ligands could be produced. The most promising approach may be the use of an array of ligands each of which may fulfil these criteria. Pattern recognition could specifically identify the organism, as has been described for lectin, glycoconjugate and peptide based ligands (Cole *et al.*, 1984; Williams *et al.*, 2003; Tarensenko *et al.*, 2004). The disadvantages of the approaches used previously are that the target to which the ligand binds has not been reported or characterised, thus it may not be ideal for detection purposes. This study has determined a number of targets for which some of the desirable properties of a detection target may be met and will be fully evaluated in future work.

A number of studies have been conducted into the elucidation of the proteins of *B. anthracis* spores (Sylvestre *et al.*, 2002, 2005; Steichen *et al.*, 2003; 2005; Lai *et al.*, 2003; Liu *et al.*, 2004; Redmond *et al.*, 2004). However, large numbers of putative spore proteins still remain undetected (Lai *et al.*, 2003; Liu *et al.*, 2004). This study has contributed to the knowledge of components of the *B. anthracis* spore and identified a number of immunogenic proteins although the location, function and potential interactions of such proteins remains unclear. The identification of a unique antigen of the *B. anthracis* spore has proven particularly challenging and to date no such antigen has been described in the literature. All previous research has focused on the use of whole spores to identify specific ligands that may or may not be suitable for detection.

In conclusion, the use of a rational design process for the production of specific ligands has been shown for the first time and used for the elimination of cross reactivity to a known protein target. Combined with the use of continuous flow sonication, a highly sensitive method for the near real time specific detection of *B. anthracis* spores has been demonstrated.

## Appendix 1 Supplementary tables

Type of biosensor and examples	Principle	Detection elements	Examples of applications (key references)
<b>OPTICAL (Evanescent wave based)</b>			
Surface Plasmon Resonance (SPR).	SPR occurs when monochromatic plane-polarized light hits a metal film, commonly gold, under conditions of total internal reflection. When this takes place free electrons of the metal oscillate and absorb energy at a certain angle of incident light. The angle at which this happens is called the SPR angle and will change due to alterations in the evanescent field i.e. binding of target to ligand.	Recognition element most commonly antibodies.	Proteins, bacteria, viruses, DNA hybridization. (Medina <i>et al.</i> , 1997.)
Light Scattering SPR.	SPR coupled to light scattering signals observed from bound particles.	Recognition element most commonly antibodies.	Bacteria, viruses, protein. Example <i>Escherichia coli</i> . (Perkins and Squirrel, 2000)
Surface Plasmon Diffraction.	Based on SPR but a surface is generated with functional (binding) and reference areas to create a surface grating structure. The amplitude of the dielectric grating is changed by binding of the target analyte to the functional areas. Binding is monitored as a change in the diffraction intensity. Any bulk effects often observed with evanescent wave techniques which take place over both the reference and functional areas are not observed as they will not alter the grating amplitude and hence not change the diffraction intensity.	Recognition element most commonly antibodies.	Proteins, bacteria, viruses. Example Human gonadotropin. (Yu and Knoll, 2004).
Surface Plasmon Field-enhanced fluorescence spectroscopy.	Based on SPR but the evanescent wave is utilised to excite fluorophores near a metal/dielectric surface.	Recognition element most commonly antibodies.	Proteins, bacteria, viruses, DNA hybridization. (Ekgasit <i>et al.</i> , 2004).

Table A1.1. Examples of biosensor technologies described on the basis of the method of transduction.

Example	Principle	Detection elements	Examples of applications (key references)
Resonant Mirror.	Through the use of a high and low index dielectric material, at a certain angle of incidence light is coupled into the high refractive index layer and undergoes total internal reflection allowing an evanescent wave to propagate to the sample layer. Reflection of the light incurs a phase change which can be monitored. The phase change is dependent on alterations in the evanescent field i.e. an analyte binding to a ligand.	Recognition element most commonly antibodies.	Proteins, bacteria, viruses, DNA hybridization. (Watts <i>et al.</i> , 1994).
Bidiffractive grating sensor.	Binding of target analyte is detected through a change in the refractive index within the evanescent field and a change in the refractive index of each propagating light mode ( $TM_0$ and $TE_0$ ). The difference between the two decoupled beam angles can be used to measure the response.	Recognition element most commonly antibodies.	Staphylococcal Enterotoxin B, Ricin. (O'Brien <i>et al.</i> , 2000).
Interferometer.	Based on an optical system of a <i>Young's</i> interferometer. Mass coverage (binding) causes a local change in the phase velocity of light in the wave-guide and is detected by an interferometric set-up.	Recognition element.	Bacteria (Svenson, 1993.)
Ellipsometry.	Measurement of a change in the polarisation state of an elliptically polarised beam from thin films	Recognition element.	<i>Salmonella Typhimurium</i> . (Schneider <i>et al.</i> , 1997.)
Imaging Ellipsometry.	Based on ellipsometry but an image of the film can be obtained making it useful for micro array.	Recognition element.	<i>Yersinia enterocolitica</i> . (Bae <i>et al.</i> , 2004).

Table A1.1. continued

Example	Principle	Detection elements	Examples of applications (key references.
Whispering gallery modes in microspheres.	Light is coupled from an optical fibre to stimulate modes in a dielectric sphere. On binding to the sphere there is an interaction with the evanescent field of the whispering gallery mode shifting the frequency of the mode.	Recognition elements could be used.	Bovine serum albumin (BSA). Arnold <i>et al.</i> , 2003
Fibre Optic.	Evanescent wave fibre optic based e.g. RAPTOR.	Recognition element most commonly antibodies.	Ricin, <i>Bacillus globigii</i> ( <i>Bacillus subtilis</i> var. <i>niger</i> spores, <i>F. tularensis</i> .. Anderson <i>et al.</i> , 2000.
<b>FLUORESCENCE BASED</b>			
Flow Cytometry.	Cells are labelled using a fluorescent dye or labelled antibody. As cells pass through the illuminated sensing region of the flow cell, a fluorescent pulse is emitted for labelled species. Scattered light from cells can also give size, shape and structure information.	Recognition element most commonly antibodies.	<i>E. coli</i> and <i>Staphylococcus aureus</i> . Gunasekera <i>et al.</i> , 2000.
Capillary electrophoresis.	Electrophoresis within a narrow bore capillary, detection is based on the output of an electropherogram which will measure UV absorbance or fluorescence.	Recognition element DNA or antibody.	Range of target analytes e.g. toxins. Bossi <i>et al.</i> , 2000.
Real-time PCR.	Detection of fluorescence emitted by dyes released during DNA amplification.	None.	DNA. Ke <i>et al.</i> , 2000.
FISH.	DNA hybridisation to a target probe labelled with a fluorophore.	DNA.	DNA. Amann and Kuhl, 1998.
Intrinsic fluorescence of biological particles.	Detection of the intrinsic fluorescence emitted by Biological particles.	None.	Intrinsically fluorescent species. Reyes <i>et al.</i> , 1999.
Vertical Cavity Surface Emitting Lasers	Detection of near infrared dyes.	None.	Thrush <i>et al.</i> , ??)

Table A1.1. continued



Example	Principle	Detection elements	Examples of applications (key references).
Bioluminescence. Adenosine triphosphate (ATP)/ Adenylate kinase (AK) detection, Luciferase based	Uses the ATP dependent luciferase-luciferin reaction to produce light. AK can be used to enhance assay sensitivity. Bacteriophage can be used for specific lysis of target organisms.	Bacteriophage.	<i>Bacteria e.g. S. aureus.</i> Prosser, 1994 and Blasco <i>et al</i> 1998
Chemiluminescence. Examples luminol, lophine, lucigenin or oxalate derivative	These can undergo oxidation in the presence of hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> ) to produce light.	Recognition element most commonly antibodies.	Range of analytes e.g. <i>E. coli</i> 0157:H7. Liu <i>et al.</i> , 2003
Electrogenerated chemiluminescence	Using a positively biased electrode luminol is oxidised in the presence of H <sub>2</sub> O <sub>2</sub> light emission occurs.	Recognition element commonly a labelled antibody.	<i>B. anthracis</i> spores, <i>S. typhimurium</i> , <i>E. coli</i> 0157:H7. Yu and Stopa, 1996.
<b>CHROMOGENIC</b>			
Enzyme Linked Immunofiltration Assay*	A biosensor based on a solid phase Enzyme Linked Immunosorbent Assay (ELISA).	Recognition element most commonly antibodies/DNA hybridization.	<i>E. coli</i> Paffard <i>et al.</i> , 1996; Aubert <i>et al.</i> , 1997.
Flow through capillary enzyme immunoassay*	A biosensor based on a solid phase ELISA.	Recognition element most commonly antibodies	SEB, bacteriophage M13, <i>E.coli</i> . Koch <i>et al.</i> , 2000.
Liquid crystals	Changes in the orientation of liquid crystals on binding of analyte can be amplified and transduced into a optical signal visible to the naked eye.	Recognition element	Biotin/avidin. Gupta <i>et al.</i> , 1998
Stabilised Colloidal systems e.g. gold hydrosols	The absorption band of gold nano-particles is typically around 520 nm on aggregation and coupling reactions that occur using a ligand and an analyte. On binding there is a shift in the surface plasmon absorption resulting in a observable colour change.	Recognition elements.	Lectin. Hone <i>et al.</i> , 2003.

Table A1.1 continued.

Example	Principle	Detection elements	Examples of applications (key references.)
Holographic. Metabolite holographic biosensor.	Change in pH due to a specific enzyme/substrate reaction using hydrogels co-polymerised with monomers incorporated with ionisable groups and cross-linkers. A change of pH produces a visible colour change.	None.	Metabolite detection. Marshall <i>et al.</i> , 2004.
<b>ELECTROCHEMICAL</b> Light Addressable Potentiometric.  Potentiometric.  Amperometric.	Measures local changes in an alternating photocurrent generated using a rapid pulsing light source (often a light emitting diode).  Electrodes detect changes in potential at constant current (usually zero).  Uses enzyme catalysed electro-oxidation or reduction.	Recognition element most commonly antibodies/DNA hybridization.  Recognition element  Recognition element most commonly antibodies.	<i>Nesseria meningitidis</i> , <i>Yersinia pestis</i> . Libby and Wada, 1998/ Hafeman <i>et al.</i> , 1998  <i>Cryptosporidium</i> . Wang <i>et al.</i> , 1997.  <i>Bacillus subtilis</i> spores. Uithoven <i>et al.</i> , 2000.
<b>PIEZOELECTRIC (GRAVIMETRIC)</b>			
Quartz Crystal Microbalance.	The surface of the crystal is coated with a specific binding molecule, binding of target analyte causes an increase in the mass and hence increase the resonant frequency of oscillation decreases.	Antibody/Aptamers.	<i>E. coli</i> , <i>Y. pestis</i> and <i>Salmonella dysenteriae</i> . Koenig and Gratzel, 1993; Liss <i>et al.</i> , 2002.

Table A1.1 continued

Example	Principle	Detection elements	Examples of applications (key references.)
<p>Surface Acoustic Wave (SAW).</p> <p>Mechanical cantilever.</p> <p>Resonating mechanical cantilever.</p>	<p>Based on surface acoustic wave mode of propagation. These waves possess a longitudinal and a vertical shear component that can couple with medium at the device interface which alters the amplitude and velocity of the wave. Therefore SAW sensors are sensitive to changes in mass.</p> <p>Binding of target analyte to ligand immobilised onto the cantilever is detected as adsorption. Stress bends the cantilever either away from or towards the analyte this can be measured using the optical beam deflection technique.</p> <p>Binding of target analyte alters the natural resonant frequency of the mechanical oscillator due to a change its total mass on binding.</p>	<p>Recognition element</p> <p>Recognition element.</p> <p>Recognition element.</p>	<p>DNA hybridization. Wu <i>et al.</i>, 1990.</p> <p>DNA. Zhang and Xu., 2004</p> <p>Baculovirus. Ilic <i>et al.</i>, 2004.</p>
<p>Magneto-electronic Magnetoresistive-based sensor.</p>	<p>Based on the giant magnetoresistive effect. Two magnetic layers are used and a magnetic field applied. When the two layers are aligned the resistance is low and when they are anti-parallel in alignment the resistance is high. Either the target analyte or secondary ligand to the target can be magnetically labelled. Recognition elements are immobilised onto magnetic field sensors which detect the presence of the magnetic label.</p>	<p>Recognition element most antibodies/DNA hybridization.</p>	<p>DNA. Graham <i>et al.</i>, 2004).</p>

Table A1.1 continued

Example	Principle	Detection elements	Examples of applications (key references.)
Magnetoelastic.	Detection of changes in the resonant frequency of the vibration of the sensor due to an externally applied magnetic field. As the mass or elasticity changes on binding of a target analyte the resonant frequency changes. These changes can be detected using a number of methods (e.g. optically or acoustically).	Recognition element.	<i>E. coli</i> 0157:H7. (Ruan <i>et al.</i> , 2003).
Electrical Impedance/Capacitance/Conductance. Ion channel.	A gated ion channel is used for signal transduction, the conductance of the ion channels switches on analyte binding, this has also be coupled to a change in optical fluorescence on analyte binding.	Recognition elements.	Cholera toxin. (Cornell <i>et al.</i> , 1997; Song and Swanson, 1999).
<b>SPECTROSCOPY</b>			
Raman.	Vibrational spectroscopy.	None.	(Nelson <i>et al.</i> , 1992)
Surface-enhanced Raman Spectroscopy.	As Raman but using modified surface to enhance the Raman signal.	None.	<i>E. coli</i> . (Jarvis and Goodacre, 2004)
Infrared spectroscopy.	Absorbance spectra can be obtained from bacteria, however, this is difficult to interpret.	None.	Rossi and Warner, 1985
Transmission Surface Plasmon Resonance Spectroscopy.	Measurement of change in surface plasmon absorbance of discontinuous gold films.	Recognition element.	Biotin/avidin. Lahav <i>et al.</i> , 2004

Table A1.1 continued

## **Appendix 2 Names and Addresses of suppliers**

**Agilent Techolgies UK Ltd**, Lakeside, Cheadle Royal Business Park., Stockport, Cheshire, UK

**Alta Bioscience** The University of Birmingham, Edgbaston, Birmingham, UK

**Anthos Labtec Instruments**, GmbH, Lagerhausstr. 507, 5071, Salzburg, Austria

**Amersham BioSciences**, Chalfont St. Giles, Bucks, UK

**BIAcore**, Rapskatan 7, Uppsala, Sweden

**BioRad**, BioRad House, Maylands Avenue, Hemel Hempstead, Herts, UK  
Cepheid

**Eurogentec Ltd**. P.C. House, 2 South street, Hythe, Southampton, Hampshire, UK  
ENI Rochester, NY

**Invitrogen**, Fountain Road, Inchinnan Business Park, Paisley, UK.

**Harvard Apparatus** (a division of Harvard Bioscience), 84 October Hill Road, Holliston, MA, USA.

**Misonix**, 1938 New Highway, Farmingdale, NY 11735.

**Molecular Devices Ltd**, 660-665 Eskdale Road, Winnersh Triangle, Wokingham, Berks, UK

**MWG Biotech**, 90 Long Acre, Covent Garden, London, UK

**New England BioLabs (UK) Ltd**. 75/77, Knowl Piece, Wilbury Way, Hitchin, Herts, UK

**Nunc**, BRL, Life Technologies Ltd., Trident House, Washington Road, Paisley, UK

**Oxoid Ltd**, Wade Road, Basingstoke, Hampshire, UK

**Oswell**, Osewill DNA sequencing Services, Southampton University, Southampton UK

**Perbio** (previously Pierce), Century House, High Street, Tattenhall, Cheshire, UK  
PHLS, 61 Colindale Avenue, London.

**Sigma**, Fancy Road Poole, Dorset, UK.

**Thermo Labsystems**, Saxon Way, Bar Hill, Cambridge, UK

**Vernitron**, Thornhill, Southampton, UK

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