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Rapid Onsite Assessment of Spore Viability

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Abstract

This one year LDRD addresses problems of threat assessment and restoration of facilities following a bioterror incident like the incident that closed down mail facilities in late 2001. Facilities that are contaminated with pathogenic spores such as *B. anthracis* spores must be shut down while they are treated with a sporicidal agent and the effectiveness of the treatment is ascertained. This process involves measuring the viability of spore test strips, laid out in a grid throughout the facility; the CDC accepted methodologies require transporting the samples to a laboratory and carrying out a 48 hr outgrowth experiment.

We proposed developing a technique that will ultimately lead to a fieldable microfluidic device that can rapidly assess (ideally less than 30 min) spore viability and effectiveness of sporicidal treatment, returning facilities to use in hours not days. The proposed method will determine viability of spores by detecting early protein synthesis after chemical germination.

During this year, we established the feasibility of this approach and gathered preliminary results that should fuel a future more comprehensive effort. Such a proposal is currently under review with the NIH. Proteomic signatures of *Bacillus* spores and vegetative cells were assessed by both slab gel electrophoresis as well as microchip based gel electrophoresis employing sensitive laser-induced fluorescence detection. The conditions for germination using a number of chemical germinants were evaluated and optimized and the time course of protein synthesis was ascertained. Microseparations were carried out using both viable spores and spores inactivated by two different methods. A select number of the early synthesis proteins were digested into peptides for analysis by mass spectrometry.

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Abbreviations

1D/2D	one dimensional/ two dimensional
BW	biological warfare
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
CGE	capillary gel electrophoresis
DPA	Dipicolinic acid
IEF	isoelectric focusing
LIF	laser-induced fluorescence
UV	ultraviolet
OD	optical density
MW	molecular weight
PCR	Polymerase Chain Reaction
Da	Dalton, unit of molecular weight used when describing proteins (1 Da = 1 atomic mass unit)
LC-MS	Liquid Chromatography Mass Spectrometry
MALDI-TOF	Matrix assisted Laser Desorption Ionization Time of Flight (Mass spectrometry)

1. INTRODUCTION

It is well accepted that fast and accurate identification of a pathogen is extremely important. However, it is equally important to assess the viability of a pathogen, especially from the standpoint of quarantine and remediation. It is also important for other scenarios including deliberate hoax events using non-viable organisms for the purpose of impacting economics and critical infrastructure. The ability of a spore to germinate is the ultimate criterion for viability and the current assay techniques (based on cell culture) are time-consuming and are not amenable to automation and portability.

Non-culture related efforts at assessing viability have been reported in the literature in recent years. Membrane active pumping, which focused on the spore membrane potential, was determined to be an unreliable indicator of viability (1). Another recent approach involves monitoring respiratory activity of spores based on the enzymatic reduction of a fluorophore (1). While successful in determining viability, this process was very slow, requiring up to 24 hours for the spores to fully germinate into vegetative cells in the presence of growth media and to begin producing enzyme. A number of rapid fluorimetric assays to assess viability have been discussed in the literature; typically, the viability of spores is assessed based on the porosity of the membranes to specific dyes. In a typical two-dye approach, one dye binds to all cells and spores while the other binds to only nucleic acids available if the spore membrane is compromised. A recent paper described the incorporation of this approach into a more quantitative ELISA based assay (2). The challenge with these approaches relates to the fact that porosity of membrane is not an absolute/reliable indicator of non-viability.

This project addresses exactly that ability to germinate, but does so in a manner that is much more rapid than waiting for cell culture and colonies to grow on a plate. Instead, the earliest stages of germination can be examined, namely the initial *de novo* protein synthesis that takes place in the first few minutes after germination. Preliminary experiments carried out in-house show that dramatic changes in the proteomic signature take place less than 1 hour after germination with l-alanine (see figure 1) and literature studies have shown that as many as 65 proteins are produced in the first 10 minutes of the germination process (3). This suggests that if we can assess changes in the protein population associated with *de novo* protein synthesis in a rapid manner, then we will have a tool that can assess viability in minutes rather than the days required for grow-out studies. Microseparations represent just such a method to rapidly probe changes in protein population, and are capable of carrying out the separation and simultaneous quantitation in typically less than 5 minutes.

The goal of this project was to develop methods and technology that would monitor and detect the very earliest stages of germination by monitoring *de novo* protein synthesis in spores that have been exposed to a chemical germinant. This project was geared towards the ultimate implementation in a fieldable device for rapid pathogen identification and viability assessment. It would be of benefit to both the emergency response to a BW event and the development and testing of new decontamination strategies and reagents (e.g. foams).

This effort will strengthen the overall biodefense portfolio of Sandia National Laboratories. Sandia has a strong presence in the areas of pathogen identification however, little if any research is geared towards tools for assessing viability. Polymerase chain reaction (PCR) based techniques, the basis of many biosafety platforms (e.g. BioWatch and Biobriefcase) are very sensitive and specific but cannot discriminate between live and dead cells. Successful development of this project would form the basis for the development of a stand-alone device for viability determination, which will complement and be compatible with other microfluidic biosensing platforms under development.

We proposed to demonstrate/develop the capability to use proteomic signatures to both identify pathogens, specifically *B. anthracis*, and to determine viability. What we envisioned for the ultimate application is a fieldable device that comprises a microfluidic protein separation and detection platform and a microfluidic sample interface capable of collecting spores, subjecting them to a heating step to eliminate any contaminating vegetative bacteria, trapping them in an appropriate chamber for treatment and incubation with an appropriate chemical germinant, and lysing and solubilizing the spores. In order to evaluate changes associated with germination, the sample would be split in half. One portion the sample would go to solubilization and direct proteomic analysis. The second portion would be treated with the chemical germinant. A fixed period later, the germinating spores would be similarly lysed, solubilized, and sent for proteomic analysis. Ten to thirty minutes should be sufficient for protein expression levels to have altered as a function of germination (1). Both the evidence of a significant alteration in response to a known chemical germinant as well as the modified protein fingerprint information itself would be used to provide a rapid and valid indicator of viability and dramatically faster than standard culture techniques.

2. EXPERIMENTAL APPROACH AND RESULTS

2.1 Spore growth and purification.

Bacillus anthracis delta Sterne, *Bacillus cereus* and *Bacillus subtilis* were each introduced into 1L Growth Medium (see Table 1) at an OD₆₀₀ of ~0.1. The cultures were grown with shaking (250 rpm) at 37°C for ~4 h. Once an OD₆₀₀ of ~0.8 was reached, corresponding to mid log growth, the cells were harvested by centrifugation (10,000 x g for 10 min), resuspended in 2L Sporulation Medium, and the new cultures shaken (200 rpm) at 37°C for ~48 h. Spores were harvested by centrifugation (10,000 x g for 10 min) and separated from cell debris through a series of salt and detergent washes [1M KCl + 0.5M NaCl, then 50mM Tris-HCl + 1M NaCl + 0.05% SDS, then TEP buffer] followed by three water washes; all wash centrifugations were at 15,000 x g for 10 min. Washed spores were sonicated twice at 20kHz for 30 seconds, to increase spore yields. Finally, the spores were further purified using a Histodenz gradient. Purified spores were resuspended in water and stored at 4°C.

2.2 Spore germination.

Purified spores were heat-activated (80°C for 10 min), harvested by centrifugation (15,000 x g for 1 min), and resuspended in water or 60mM dipicolinic acid (DPA) + 60mM CaCl₂ (needed to maintain solubility of DPA), to an OD₆₀₀ of ~1.0. The spores were then incubated at 37°C. At the times indicated (5, 20, or 60 min) the spores were re-isolated by centrifugation (15,000 x g for 1 min) and immediately prepared for electrophoresis (outlined below).

Spores were rendered non-viable by two different methods for the purposes of this project: 1) steam sterilization by autoclaving (121°C for 30 minutes) or chemical sterilization (30 minute exposure to 10% (v/v) hypochlorite bleach, followed by a brief wash step with water). After treatment, the spores were verified non-viable by overnight culture.

2.3 SDS-PAGE.

Spore samples were resuspended in 2x Tris-Glycine SDS Sample Buffer (Invitrogen), 1/10 volume of 10x NuPAGE Reducing Agent was added, and the samples were heated at 85°C for 2 min. The samples were loaded onto a 10-20% Novex Tris-Glycine gel (Invitrogen) and run at 150V for 80 min. Proteins in the gel were stained with Coomassie blue, using standard procedures. Alternatively, spore samples were resuspended in 50 mM borate buffer containing 1% SDS and 50 mM tris(2-carboxyethyl)phosphine (TCEP) reducing agent which interfered with subsequent microscale separation methods less than the traditional β-mercaptoethanol reducing agent of the NuPAGE product. Comparable gel patterns were observed for both solubilization protocols in side-by-side experiments (data not shown).

2.4 Microscale Electrophoretic Separations

Duplicate samples prepared above were diluted, typically 1:1 with CGE buffer (5 mM borate, pH 8.5 with 5 mM SDS) and centrifuged briefly to pellet any precipitate. The supernatant was then applied to a BioSpin P6 column (BioRad) previously equilibrated with CGE buffer, in order to remove the TCEP. Samples eluted from the Biospin columns were then treated with a 10% final volume of 10 mM fluorescamine and mixed on a vortexer for 30 seconds prior to injection onto the μChemLab platform. Red fluorescently labeled proteins were added to all samples to act as internal standards. Conditions for separation and detection were as previously described (ref).

2.5 Mass spectrometry.

Protein bands of interest were excised from the gel, destained using standard procedures, and digested with trypsin at 37°C for 16 h. The peptide products of these digests were extracted from the gel using 50% acetonitrile + 5% formic acid, and concentrated under vacuum. A 1 µL portion of the sample was mixed 1:1 with 6.2 mg/ml α-cyano-4-hydroxycinnamic acid in 36/56/8 methanol/acetonitrile/water (Agilent Technologies, Palo Alto, CA) for analysis by MALDI-MS. A 5 µL portion of the sample was used for LC-MS/MS.

Table 1: Buffer Compositions

Buffer/Medium	Quantities	
Growth Medium	ml	
Stock I + II	940	
Stock III	50	
Stock IV	2	
Stock V	10	
Stock I+II	grams	grams
L-glutamic acid (pH 7.0)	3.9	15.6
Casein hydrolysate	10.6	42.4
L-alanine	1.3	5.2
L-asparagine	1.5	6.0
KH ₂ PO ₄	1.4	5.6
NH ₄ Cl	1.4	5.6
Na ₂ SO ₄	0.12	0.48
NH ₄ NO ₃	0.11	0.44
FeCl ₃ · 6H ₂ O	0.001	0.004
Total Volume	1 L	4 L
Stock III		
MgSO ₄ · 7H ₂ O	0.99 g	1.98 g
10% (w/v) CaCl ₂	2 ml	4 ml
Total Volume	500 ml	1 L
Stock IV		
MnSO ₄ · 4H ₂ O	1.1 g	5.5 g
Total Volume	100ml	500 ml
Stock V (filter sterilize)		
L-tryptophan	0.2 g	2.0 g
Total Volume	100ml	1 L

Table 1 *Continued*

Sporulation Medium		
dH ₂ O		890 ml
Sol'n A		900 μ l
Sol'n B		9 ml
Sol'n C		40 ml
Sol'n D		10 ml
Sol'n E		40 ml
Sol'n A (filter sterilize)		grams
FeCl ₃ · 6H ₂ O		0.089
MgCl ₂ · 6H ₂ O		0.830
MnCl ₂ · 4H ₂ O		1.979
Total Volume		100 ml
Sol'n B (pH 7.0) (filter)		grams
NH ₄ Cl		53.5
Na ₂ SO ₄		10.6
KH ₂ PO ₄		6.8
NH ₄ NO ₃		9.7
Total Volume		1 L
Sol'n C (5% L-glutamate)		
L-glutamate (pH 7.0)		3.7 g
Total Volume		500 ml
Sol'n D (0.1M CaCl ₂)		
CaCl ₂		5.6 g
Total Volume		500 ml
Sol'n E (1M MgSO ₄ · 7H ₂ O)		
MgSO ₄ · 7H ₂ O		3.7 g
Total Volume		500 ml

3. RESULTS AND DISCUSSION

3.1 Spore Germination Studies:

We have focused on *B. anthracis* (the avirulent delta Sterne strain) and its near neighbors of *B. subtilis* and *B. cereus* for this work. These organisms are currently in culture in our labs and all three species sporulate. It is important to note that although we are using an avirulent version of the *B. anthracis* for these pilot studies, its spore forming and germination abilities are intact.

The first step was to evaluate proteomic signatures of both the vegetative cells and the spores. Figure 2 shows typical images of 1D protein sizing gels comparing vegetative cells and spores of the same species. There are clear protein pattern differences between spores and the corresponding vegetative cells. The spores of the three *Bacillus* species examined typically display a larger proportion of small molecular weight proteins, corresponding to, among others, the high copy number spore coat proteins and this is comparable to what has been observed in the literature for these species (4, 5).

The next stage of the project was to optimize the germination process for this application. The standard of evaluation was the degree of altered protein pattern in response to the chemical germinant and the ability to distinguish spores that have begun *de novo* protein synthesis. A number of chemical germinants were evaluated including alanine for *Bacillus anthracis* (delta Sterne) and *Bacillus subtilis* and inosine for *Bacillus cereus*. Viable spores were first heat shocked to both inactivate any vegetative cell matter contaminating the spores as well as to help stimulate spores. The spores were incubated with different concentrations of either alanine or inosine (10 to 250 mM) and then solubilized and separated by slab gel electrophoresis. Dipicolinic acid (DPA), a chemical produced by germinating cells and a known stimulant of *Bacillus* spores (6) was also evaluated and, as shown in Figure 3, found to be much more effective than the discrete amino acid germinants, yielding more distinct new protein synthesis peaks sooner relative to spores in the absence of germinants.

It was next necessary to determine the minimum amount of time required for distinct discrimination between viable germinating spores and non-germinating spores. This is extremely important to the ultimate “rapid analysis” application of this work. Time courses were evaluated covering the range from 0 to 120 minutes. As can be seen in Figure 4, for both *B. anthracis* (delta Sterne) and *B. cereus*, the changes are apparent in as little as 5 minutes with DPA as the germinant. Indeed, the bulk of the changes appear to take place in approximately 5 minutes, with little more change happening from between 5 and 60 minutes. This suggests that the early protein germination events do indeed take place rapidly as suggested in the work by Hirano (3). In reality, there is a larger error expected to be associated with the shorter incubation times, simply because of the finite amount of time required to handle the sample after incubation (eg. 1 minute centrifugation step, with possibly 1 minute acceleration/deceleration times). Regardless of the precise timing, a 5 to 10 minute incubation time is clearly sufficient to generate actionable protein signature differences and is well within a reasonable timeframe for the proposed application.

Temperature during the germination process was also evaluated and, not surprisingly, germination at elevated temperatures (37⁰C) was found to be somewhat more effective than at room temperature for a fixed period of incubation (data not shown). As a result all subsequent incubations were carried out at 37⁰C. Such an elevation of temperature is an easy feature to incorporate in the ultimate micro system and will not have a negative impact on the ultimate implementation.

It is important to note that in all cases, these germination experiments were carried out in the absence of culture media. Culture media is undesirable from a device implementation standpoint for a number of reasons: 1) it can be expected to clutter the protein signature, requiring that the spores be thoroughly washed prior to analysis, and 2) it represents an additional reagent, one that requires certain storage conditions to maintain efficacy. While the addition of culture media was evaluated (data not shown), it was determined that it was not necessary for the generation of usable protein pattern changes. The reservoir of amino acids present in the spores themselves was apparently sufficient to allow generation of new proteins, at least in the short time frame needed for DPA germination. The ability to perform the germinations in the absence of culture media is a great benefit to the ultimate application, eliminating the need to wash spores after germination to prevent masking of protein changes due to the high (and relatively constant) protein concentrations of growth media.

The critical question for this application is whether the germinating spores generate actionable alterations in protein signatures, and can be used to determine the viability of the spores. As a result, the critical control experiments involving non-viable spores needed to be carried out. Spores were inactivated by both heat (autoclaving) and chemical means (bleach). In both cases, the spores were fully inactivated as evidenced by a lack of colony formation upon culturing (data not shown). As seen in Figure 5, the signatures for inactivated material did appear different from viable spores in the case of autoclaved spores and dramatically different from viable spores in the case of bleached spores. The latter observation is most likely due to a loss of structural integrity resulting from bleaching. In either case the inactivated spores showed little difference upon addition of the germinant. This is the critical result needed to validate the feasibility of this approach. In addition, the altered protein patterns are expected to provide additional confirmation of the non-viability of spores, at least by the two methods evaluated here. Additional inactivation methods will need to be explored for their impact on the spore protein signatures.

3.2 Microscale Separation Technologies

A key aspect of rapid determination of spore identity and viability is the ability to determine the proteomic signatures rapidly and the slab gel techniques used for the prior methods development, which typically take at least 8 hours to process, are far too time-consuming and labor intensive for a potential fieldable rapid spore typing and viability device. Chip based gel electrophoresis (CGE) has proved very successful for the determination of protein signatures in previous work by this group (7). Figure 6 shows representative traces of autoclaved and viable spores exposed to the germinant DPA. Both the 5 minute and 30 minute exposure show clear differences over the dormant viable spores (exposure to H₂O) and even more pronounced differences over the autoclaved material. It is the presence of the changed signature, of the germinating spores over the non-viable spores, which is the fundamental concept of this project.

Only two inactivation methods have been evaluated here: autoclaving and bleaching. Further research should expand the list of inactivating protocols to observe any additional differences in the protein patterns specific to the inactivation protocol. Candidates should include gamma irradiation, UV irradiation as well as the common gas treatments including chlorine gas, hydrogen peroxide vapor and ethylene oxide vapor, all of which were beyond the scope of this preliminary effort. The latter gas-based chemical treatments are especially relevant to the concept of facilities remediation, as it is likely that fumigants will be used in many clean-up and remediation scenarios.

The separations shown in Figure 6, while adequate for this demonstration of feasibility, would benefit from higher resolution. The commercially available sieving gel has a fractionation range that is in the range of 14 to 200 Da. *Bacillus* spores have traditionally been poorly resolved using this gel, primarily due to the large numbers of small molecular weight spore coat proteins. While it remains to be seen how much higher resolving power will improve the ability to determine germination specific changes in protein signatures, further development of this technique should include the incorporation of a higher resolving separation medium such as was under development in the DoD funded μ ChemLab effort. Two different candidate technologies have shown promise for this. One is the development of cross-linked polymer based separation media comparable to the SDS-PAGE experiments but cast in a microchip which allows rapid microseparations (8) but with a potentially tunable pore size that could be tailored to a smaller molecular weight range more appropriate to spore analysis. The second approach would be to make use of replaceable high viscosity gels that have shown promise for reducing the overall separation time from ~300 seconds down to ~30 seconds (data not shown). Employing a longer separation channel with these replaceable gels, however, should also allow improvement of separation efficiency at the expense of longer separation times. Extremely short separation times, though attractive, are not of paramount importance for this effort since they will still be only one component of the overall process, along with the multi-minute incubation with the germinant and the subsequent sample processing steps.

Sample processing, though handled manually in this preliminary project, has also been developed under the Department of Defense funded μ ChemLab effort and it has been demonstrated that all the steps that are need to concentrate and lyse spores, solubilize and fluorescently label their proteins prior to injection into the microseparations platform can be achieved in an automated fashion in approximately 7 minutes (data not shown), appropriate for the ultimate on-site implementation of this device.

3.3 Mass Spectrometry

For the purpose of correlating emerging protein bands with known early germination proteins, select bands were chosen for further analysis by mass spectrometry. As seen in Figure 4, a number of protein bands are routinely seen as a result of the addition of the chemical germinant DPA, and are highlighted by arrows. As an example, in all three species, a high molecular weight protein, ~ 80 kDa, commonly seen in viable non-germinated spores treated only with water, was absent in viable spores treated with DPA. Another example was the appearance and growth of a smaller molecular weight species, ~30 kDa, protein band in germinating spores that was likewise absent in non-germinated spore samples. These two protein bands, along with a number of others, were isolated for mass spectral analysis. The bands were excised and were digested into peptides, using in-gel digest, for analysis by liquid chromatography mass spectrometry (LC-MS).

Peptides isolated for a given sample were then subjected to collision induced dissociation (MS/MS) to aid in sequence identification and those spectra were searched against the appropriate *Bacillus* species database for candidate protein identifications using the Mascot program (Matrixscience, Boston, MA). The results for the LC-MS/MS analysis are summarized in Table 2. For each protein band, the top three candidate protein identifications have been listed based on protein score, which is an aggregate number combining information from all individual peptides detected. Also listed are the numbers of peptides identified in the candidate protein sequences as well as the protein score generated from searching a reverse database. Both the former and the latter numbers give indications, along with the protein score itself, of the probability that the candidate protein identification is valid. A large number of peptide “hits” from a particular candidate protein sequence tends to validate the identification of that protein over another candidate with only perhaps one peptide hit detected. Likewise, a protein score should be large relative to that which would be obtained randomly as in the case of a reverse database search, in which all sequences in the database are arbitrarily reversed.

Figure 7 shows a representative MS/MS spectrum used to identify one of the peptide sequences resulting from one of the protein bands, in this case Sample 3, corresponding to the ~30 kDa band in *B. anthracis*. The top candidate identification of that protein band is a metabolic protein of *B. anthracis* Sterne, fructose-biphosphate aldolase, class II. The inset in Figure 7 shows the sequence for this putative identification and the amino acid sequences of the various detected peptides are highlighted in red. The second inset of Figure 7 shows the MOWSE score for the protein identification derived from the MASCOT search and is an indication the probability of accurate identification and is typically a hyper geometric dispersion. Small protein scores, within the green highlighted region, correspond to random matches across all possible proteins. The protein score of 212 is far removed from the random scores and as such is most likely a valid identification.

Preliminary analysis of the protein identifications reveals some interesting results. In *B. anthracis*, the ~30 kDa protein that appears in response to a germinant appears to be a metabolic protein as mentioned above and that might be expected on going from a dormant spore towards a metabolically active vegetative cell. Likewise, the ~25 kDa band that appears in response to germinants is identified as being possibly the transcription factor CodY, which also might make sense given the initiation of new protein synthesis. However, some of the potential identifications are more difficult to rationalize such as the ~20 kDa molecular weight band that appears in germinating spores has been logged as a spore coat protein, SpoE which would not be expected to be up-regulated except in *sporulating* cells. It is possible, that this increase in SpoE might reflect *release* and possible increased availability of spore coat protein from the germinating spores.

The results, while intriguing, are far from conclusive and a more extensive study is desirable. One of the primary issues in the use of MS after 1D SDS-PAGE as was performed here is the fact that 1D gel separations are insufficiently resolved to yield high quality mass spectrometric data. The problem stems from the fact that each discrete protein band can be expected to be comprised of more than one protein. This often makes it difficult to determine individual proteins because of the large number of possible peptides. This was certainly a problem in attempting to use MALDI-TOF alone rather than mass spectrometry in conjunction with the liquid chromatography step that was ultimately used to generate the data presented in this report. Two dimensional gel electrophoresis performed prior to in gel digest and MS would be expected to improve this, and will be pursued as resources allow in the future, potentially as part of a National Institutes of Health proposal currently under review.

Table 2 Mass Spectrometric Analysis of Selected Protein Bands Altered in Response to Germination

Sample	Species	Best Candidates	Protein	Calculated MW	Number of Peptide Hits	Protein Score	Reverse Database Score
2	B. anthracis	BAS0102	DNA-directed RNA polymerase, β subunit	131.809	1	53	12
		BAS0103	DNA-directed RNA polymerase, β subunit	134.365	1	47	12
9	B. cereus	BC0129	translation elongation factor Tu	42.912	3	202	8
		BC2889	inosine-uridine preferring nucleoside hydrolase	36.210	2	108	8
		BC0123	DNA-directed RNA polymerase, β subunit	134.266	1	88	8
3	B. anthracis	BAS5184	fructose-bisphosphate aldolase, class II	30.654	6	212	9
		BAS4196	conserved hypothetical protein	30.315	1	57	9
		BAS5004	conserved hypothetical protein	33.425	1	47	9
10	B. cereus	BC4047	hypothetical protein	14.059	2	106	15
		BC0122	DNA-directed RNA polymerase, β subunit	131.783	1	55	15
		BC2118	respiratory nitrate reductase, α subunit	139.336	1	45	15
4	B. anthracis	BAS3619	spore coat protein E	20.388	4	195	15
		BAS2771	conserved hypothetical protein	27.998	3	123	15
		BAS3679	transcription factor CodY	28.756	2	61	15
15	B. cereus	BC3770	spore coat protein E	20.330	5	335	13
		BC3825	SSU ribosomal protein S2P	26.546	2	97	13
		BC2967	fructose-bisphosphate aldolase, class II	27.958	2	80	13
5	B. anthracis	BAS0340	conserved hypothetical protein	19.409	2	84	15
		BAS3619	spore coat protein E	20.388	2	81	15
		BAS3681	ATP-dependent protease hslV	19.437	1	47	15
11	B. cereus	BC3770	spore coat protein E	20.330	2	113	17
		BCp0002	hypothetical protein	20.330	3	113	17
		BC4646	SASP	6.837	1	73	17
7	B. anthracis	BAS5242	conserved hypothetical protein, 97% identical to BC5391	16.286	3	154	14
		BAS0341	conserved hypothetical protein	16.818	2	81	14
		BAS3701	acyl carrier protein	8.507	1	62	14
12	B. cereus	BC4625	universal stress protein family	16.700	4	140	16
		BC3848	acyl carrier protein	8.809	2	102	16
		BC5391	hypothetical protein, 97% identical to BAS5242	16.090	1	85	16
1	B. anthracis	BAS0102	DNA-directed RNA polymerase, β subunit	131.809	5	183	26
		BAS0103	DNA-directed RNA polymerase, β subunit	134.365	2	72	26
		BAS3619	spore coat protein E	20.388	1	44	26
14	B. cereus	BC3770	spore coat protein E	20.330	4	215	12
		BC0131	LSU ribosomal protein L3P	22.692	2	73	12
		BC3823	uridylate kinase	25.883	1	67	12

4. CONCLUSIONS

This work represents a successful preliminary research study on the feasibility of using the early germination protein production of spores in response to a chemical germinant as a means of determining the viability of an organism. We have optimized the protocol for germination of *Bacillus* spores in the absence of growth media. Spores exposed to dipicolinic acid were found to generate distinctly different protein patterns in as little as 5 minutes and were able to correlate the alteration of the protein pattern to the viability of the spores. Spores made non-viable by either autoclaving or bleaching displayed different protein patterns relative to viable but dormant spores and displayed no appreciable differences in pattern in response to the chemical germinant. The existence of changed proteomic signatures in response to a specific chemical germinant, in and of itself, is the indicator of germination and, therefore, viability.

We have used microseparations, specifically chip based gel electrophoresis combined with laser-induced fluorescence detection, to rapidly demonstrate differences in patterns from viable and inactivated spores and have shown that the differences vary as a function of time.

Microseparations were complete in approximately 5 minutes; this time combined with 5 to 10 minutes incubation with the germinant and allowing 7 minutes for additional sample processing is fully consistent with the stated goal of producing a 30 minute viability determination. The successful completion of this one-year LDRD will form the foundation for further research and development. The preliminary results gained in this project have already allowed us to submit a proposal (R21) to the National Institute of Allergy and Infectious Disease. This proposal is currently under review.

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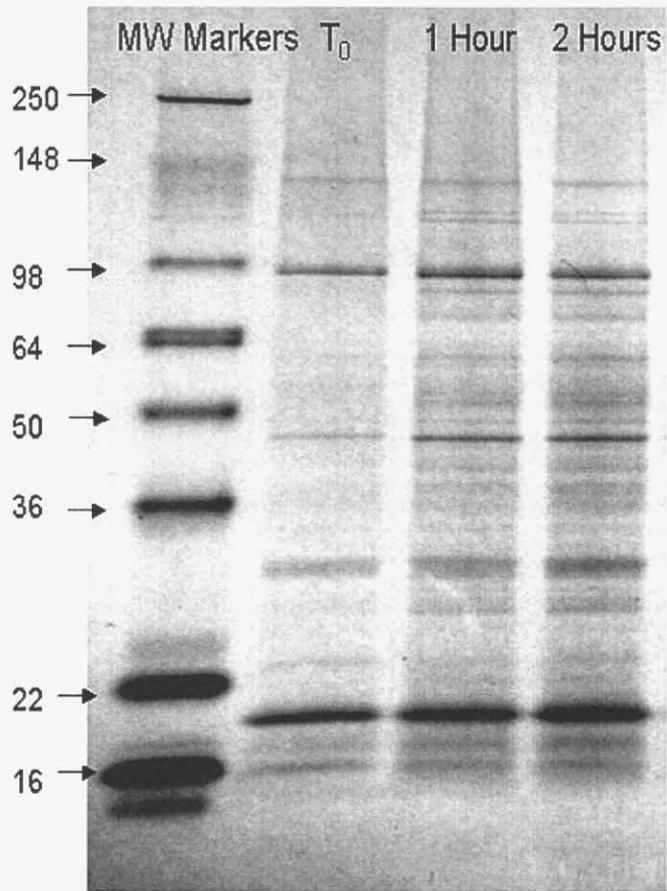


Figure 1: Preliminary results showing activation of *B. anthracis* (delta Sterne) spores using alanine. Protein patterns are clearly differentiated within an hour of exposure to germinant.

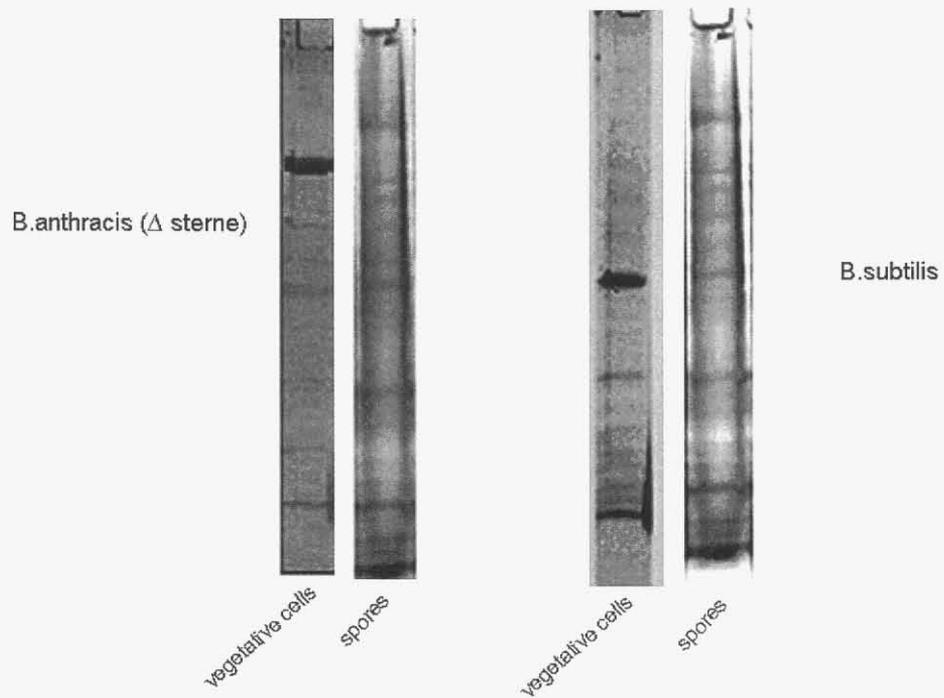


Figure 2: SDS-PAGE protein separation of viable vegetative cells and viable spores of a) *B. anthracis* (delta Sterne) and b) *B. subtilis*.

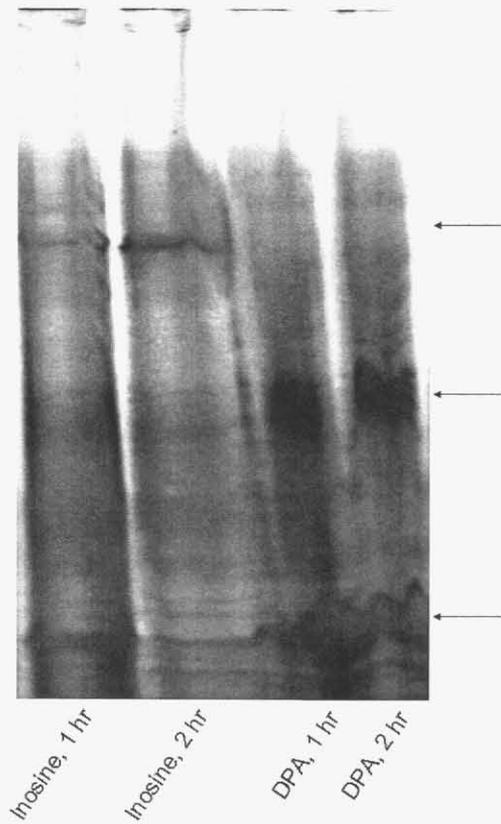


Figure 3: Optimization of spore germination conditions. Germination of *B. cereus* spores with 100 mM inosine (left two lanes) and 60 mM DPA + CaCl₂ (right two lanes). The levels of protein production in response to the germinant are much more profound in the case of the DPA than the inosine for a comparable period of time.

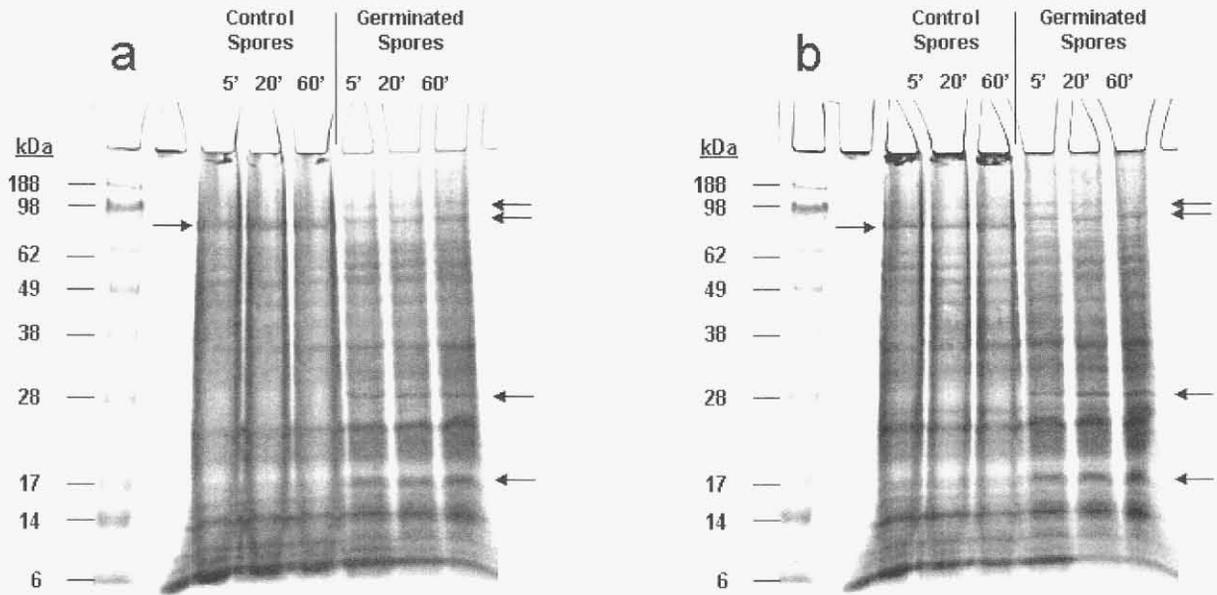


Figure 4: Proteomic fingerprint of *Bacillus* germination. *B. anthracis* (delta Sterne) a) and *B. cereus* b) spores were exposed to either water (no germinant) or DPA-CaCl₂ for 5, 20 or 60 minutes. Proteins that show clear germination associated changes in relative abundance are highlighted with arrows.

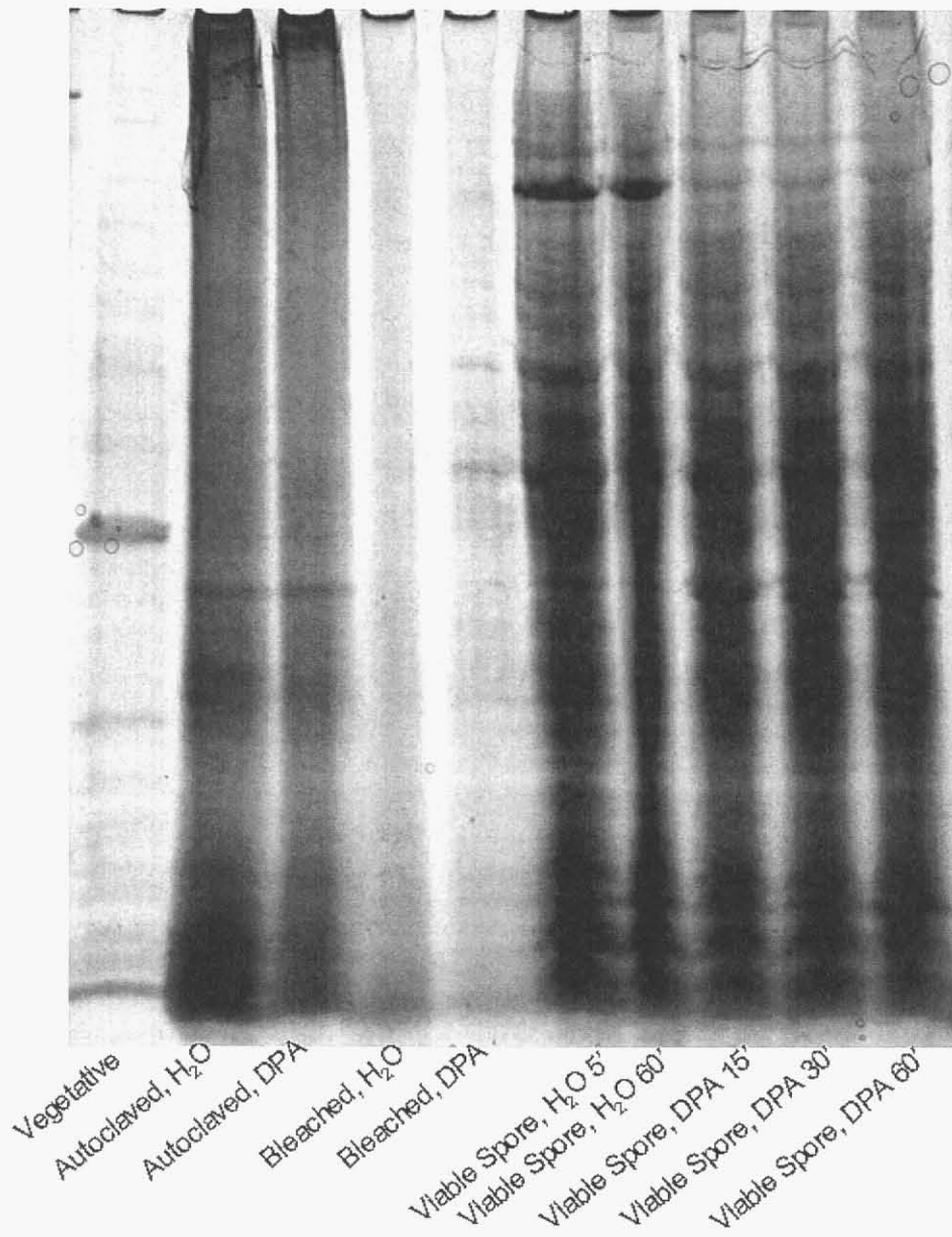


Figure 5: Comparison of behavior of viable and non-viable *B. subtilis* spores to germinants. Spores were inactivated by either autoclaving or by exposure to bleach. From left: non-viable spores (autoclaved) exposed to water or dipicolinic acid for 60 minutes; non-viable spores (bleached) exposed to water or dipicolinic acid for 60 minutes; and viable spores exposed to water for 5 minutes and 60 minutes and dipicolinic acid for 15, 30 and 60 minutes.

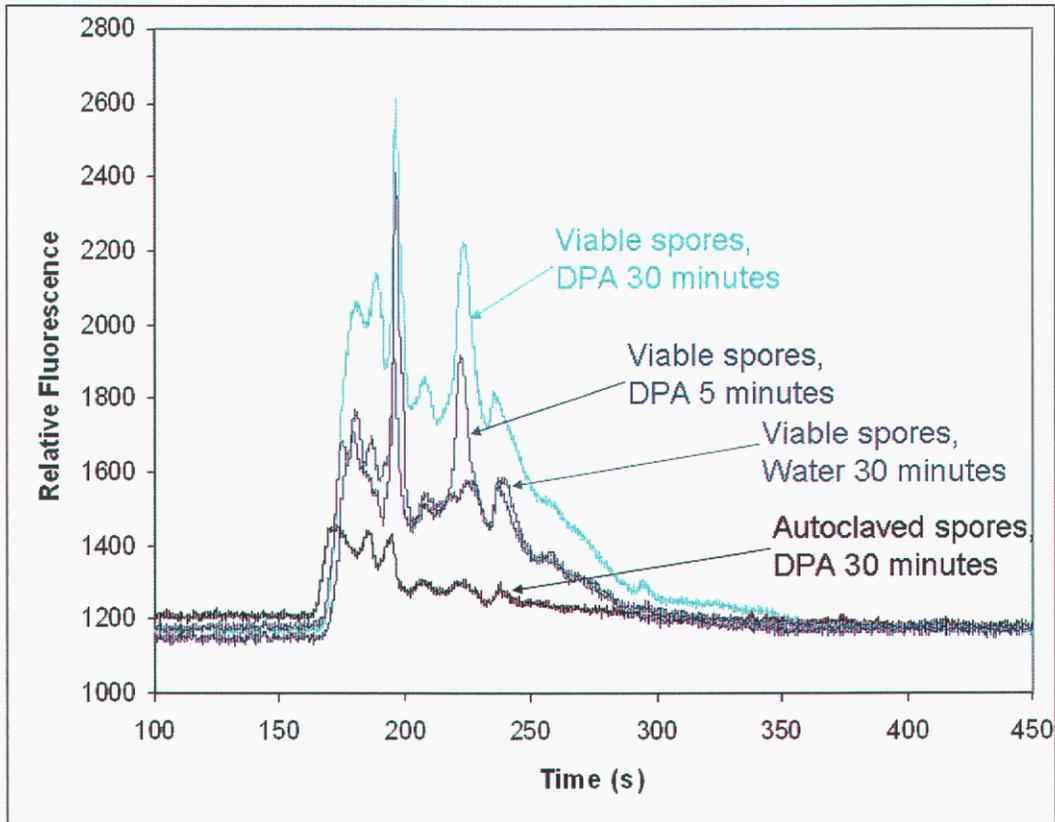


Figure 6: Chip gel electrophoretic traces of *B. cereus*. Non-viable spores (autoclaved) exposed to the germinant DPA for 30 minutes and viable spores exposed to either water (no germinant) or DPA for 5 and 30 minutes. Autoclaved spores show CGE patterns distinct from viable spores and show no apparent change in patterns in response to the germinant. Viable spores show distinct germinant-specific changes in as little as 5 minutes when compared to viable spores exposed to only water.

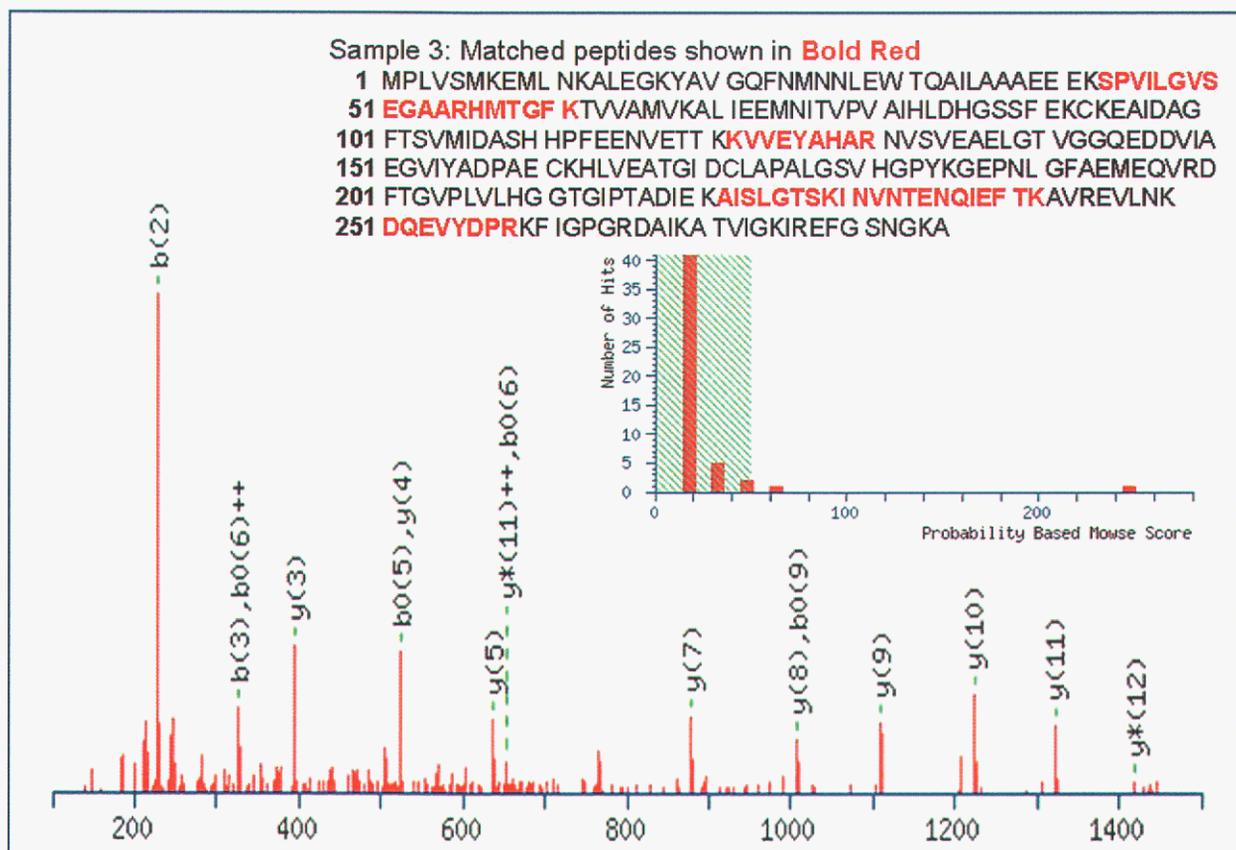


Figure 7: representative ms/ms from one of the selected bands that were altered in response to DPA. This band named Sample 3, corresponds to a ~30 kda protein of *B. anthracis* that appears in response to DPA but is absent in the non-germinated viable spore (see Figure 4).

5. DISTRIBUTION

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