Talanta 85 (2011) 1734–1737

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Establishing the detection threshold for *Bacillus subtilis* in a complex matrix using an inorganic fingerprint approach

S.M. Lev[b,*], G. Gasparich[a], F. Choi[a], L. King[b], J. Moore[b], S. Zimmerman[b]

[a] Department of Biological Sciences, Towson University, 8000 York Road, Towson, MD, 21252-0001, USA
[b] Urban Environmental Biogeochemistry Laboratory, Towson University, 8000 York Road, Towson, MD, 21252-0001, USA

ARTICLE INFO

Article history:
Received 25 May 2011
Received in revised form 24 June 2011
Accepted 27 June 2011
Available online 3 July 2011

Keywords:
Bacteria
Forensic fingerprinting
ICP-MS
Trace elements

ABSTRACT

Methods for the detection and characterization of airborne biological warfare agents, such as bacteria, using their DNA or organic composition are fairly well developed. This approach is useful for identifying the type of bacterial strain once the organism has been isolated from the matrix sampled (e.g., dust particles) and can identify genetically related organisms, which might be helpful during a forensic investigation. However, this genetic signature will not reveal information related to the methods used to grow and weaponize the organism. Bacteria will take on an inorganic signature that is related to their growth and processing history. Therefore, the ability to characterize the inorganic fingerprint of a biological particle has the potential to detect the presence of a bio-agent and expand the forensic tools available to those investigating the origin of biological weapons. This investigation builds on previous work documenting the usefulness of the inorganic fingerprint and evaluates the limits of detection in the presence of background dust. Based on ICP-MS measurements and mixing models of digested mixtures of laboratory cultured *Bacillus subtilis* (anthrax stimulant) and NIST Standard Reference Material 2709 (dust stimulant), the inorganic fingerprint method is capable of detecting toxicologically relevant levels of a bio-warfare agent in the presence of a complex background matrix.

1. Introduction

Currently, detection of biological agents is fraught with problems of too many false positives and false negatives. A major reason for these problems is the variable background of biological and non-biological particles that exist in the atmosphere. Developing technology that will be able to distinguish between background bioaerosols and bioweapon aerosols will greatly contribute to the overall chemical and biological defense capabilities of the US. Recently published work by Gikunju et al. (2004) [1] demonstrated the use of direct injection inductively coupled plasma mass spectrometery (ICP-MS) for the purpose of chemical characterization of biological materials, such as bacteria, using an inorganic chemical fingerprint. The use of ICP-MS technology offers several attractive features for trace element studies including rapid multi-element analysis and very low detection limits typically in the $pg\,g^{-1}$ to $ng\,g^{-1}$ range for most elements. This excellent sensitivity translates into superior detection limits meaning an ability to detect a very small number of spores per sample volume. Based on the generally low toxic effect threshold (i.e., 10^3–10^4 spores/L) for many biological warfare agents, the spore detection limit is a critical aspect of any approach put forth as a potential bioweapon detection technology.

Bacillus anthracis is a particularly dangerous biological agent given the low toxic effect threshold for humans (i.e., 8000–50,000 spores). Assuming a concentrated powder, a toxic dose could be delivered via the inhalation of a sub-milligram dose. Given this constraint, any method proposed for the detection of a biological agent must be extremely sensitive and flexible enough to adapt to changing background conditions. The inorganic fingerprint approach using direct injection ICP-MS first proposed by Gikunju et al. (2004) [1] seems to meet these demands. This method has not been tested in the presence of a complex matrix, such as atmospheric dust, that is likely to be present in any aerosol sample.

An estimate of the sensitivity needed to detect the presence of a biological warfare agent in aerosol form at a toxic level can be established with a simple calculation. An average adult has a lung capacity of 4–6 L and the minimum toxic effect threshold for inhaled *B. anthracis* is estimated at 8000 spores or one deep breath at the site of release [2]. Assuming a minimum lung capacity of 4 L, a toxic dose would require a spore concentration of 2 spores/cm^3 of air. If a time integrated aerosol sample were collected at this site over a 12 h period at a minimum flow volume of $30\,L\,min^{-1}$ [3] approximately 4.32×10^7 spores would be collected along with the background materials. This model requires that in order for any bio-warfare agent detection method to be most effective it must be

* Corresponding author.
E-mail address: slev@towson.edu (S.M. Lev).

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doi:10.1016/j.talanta.2011.06.072

capable of detecting the presence of less than 4.32×10^7 spores in a typical aerosol sample. A minimum effective sensitivity would be based on the higher inhalation toxicity threshold of 50,000 spores as suggested by the Centers for Disease Control (CDC) and requires the ability to detect the presence of 2.7×10^8 spores in the presence of a typical aerosol sample. The mass of background material associated with a typical aerosol sample, collected as described above, will be variable and highly dependent on geography and climate. Despite this variability, a typical sample would likely contain 10–100 s of μg of dust [3]. The inorganic fingerprint of this material in a particular geographic region will generally reflect the chemical composition of local soils and any larger regional dust sources [4]. While there will be seasonal fluctuations in the amount of particulate matter including background biologics, the chemical fingerprint of aerosol dust in a region will be predictable. Assuming a known background dust fingerprint, it may be possible to detect abrupt changes in this fingerprint that would be indicative of the release of a bio-warfare agent in a given location.

In an effort to determine the sensitivity of the Gikunju et al. (2004) [1] approach, this investigation will determine the chemical fingerprint of an anthrax stimulant (*B. subtilus*), an aerosol dust stimulant (NIST SRM 2709, San Joaquin Soil) and bacteria:dust mixtures. *B. sutilus* is the anthrax simulant preferred by both US Department of Defense (DOD) and private R&D laboratories developing detection and protection technologies [5]. *B. subtilis* is a ubiquitous bacterium commonly recovered from water, soil, and air. The bacterium produces an endospore that allows it to endure extreme conditions of heat and desiccation in the environment. Under most environmental conditions the organism is not biologically active, but exists in the spore form [6]. Although the actual numbers in existence in the environment for this species have not been determined, bacilli occur at population levels of 10^6–10^7 per gram of soil [6]. However, unless a soil has been recently amended with organic matter providing readily utilizable nutrients, the bacilli exist in the endospore stage. It is thought that 60–100% of soil bacilli populations exist in the inactive spore state [6]. Like most members of the genus, *B. subtilis* is aerobic [7]. Counted batches of bacteria will be used so that an accurate cell/dust ratio can be established and compared to the threshold calculations described previously.

2. Material and methods

2.1. Instrumentation

A Thermo Elemental (VG) PlasmaQuad ExCell inductively coupled plasma mass spectrometer in the Mass Spectrometry Laboratory at Towson University was used for these experiments. The sample introduction system consists of a variable speed peristaltic pump and a 300-place auto-sampler. The instrumental parameters are summarized in Table 1. Drift in the sample signal is common over time with the ICP-MS method, as a result, an internal standard was used to monitor and correct for instrumental drift and possible matrix effects during an analysis. In these experiments, all samples, standards and blanks were spiked with 1 ng g^{-1} In. The In detected in each sample through out the run can be compared to the In detected in the first sample (typically a blank) analyzed. A ratio of >1 required negative correction where as a ratio of <1 required a positive adjustment. Drift corrections were typically small and replicate analyzes during a run confirmed the validity of the correction.

2.2. Preparation of bacterial samples

Individual, colony populations of *B. subtilis* culture were used as the bio-agent simulant for all experiments. Cultures of *B. subtilis* were spread on a standard LB media (10 g Bacto-tryptone, 5 g yeast extract, 10 g NaCl per liter, pH 7.5) agar plate to obtain individual colonies. A single colony was then used to inoculate 500 mL of liquid LB media, which was grown for 12 h at 37 °C with aeration. Cultures were then harvested by centrifugation and bacterial pellets were washed by resuspension of the pellets in sterile, distilled water followed by recentrifugation. This pellet wash was repeated. Finally, pellets were lyophilized and stored at −20 °C until ready for analysis.

Table 1
ICP-MS operating parameters during data collection.

Instrumental parameters	
Coolant gas (L min^{-1})	12.70
Auxiliary gas (L min^{-1})	0.70
Nebulizer gas (L min^{-1})	1.0
Plasma FW power (W)	1350
Scanning parameters	
Scanning mode	Peak jump
Number of replicates	3
Dwell time (μs)	10000
Sample uptake (s)	90
Rinse time (s)	90
Sample pump rate (L min^{-1})	850
Acquisition time (s)	58
Channels per mass	3
Channel spacing	0.02
Detector sweeps	150

2.3. Quantitative evaluation of B. subtilis cultures

Samples (1 mL) were taken aseptically from each 500 mL *B. subtilis* culture prior to centrifugation for processing at the time intervals used in the study. Serial dilutions were performed (10^{-2} through 10^{-7}) in LB, and 100 μL from each tube was spread onto each of two plates of LB agar. Each sample was run in replicate. Plates were incubated at 37 °C overnight, and colonies were counted the next day for CFU/mL determination.

2.4. Sample digestion procedure

Dried bacteria and NIST SRM 2709 were prepared for analysis by ICP-MS using a similar procedure; any differences in the procedure are noted. Powdered samples were weighed directly into 30 mL screw top Teflon vials. To digest the bacteria, 4 mL of high purity 7 N HNO_3 was added to each vial, vials were then capped and placed on a hotplate at 150 °C for 24 h or until completely digested. For the NIST SRM, 3 mL of high purity HNO_3 and 1 mL of high purity HF were added to each vial and then treated as described. Once samples were digested, vials were uncapped and taken to dryness. Once the samples were dry, the residue was re-dissolved by adding 2 mL of 2% HNO_3 with 1 ng g^{-1} In. Once in solution, bacterial samples were transferred to a clean 15 mL vial and brought to a final volume of 10 mL. NIST SRM samples were transferred to a clean 125 mL bottle and brought to a final volume of 75 mL for analysis by ICP-MS.

2.5. Preparation of simulated mixtures

Solutions of digested NIST SRM 2709 and the stimulant *B. subtilis* were mixed in different proportions in order to simulate the presence of sub-milligram levels of a bio-warfare agent in the presence of background dust. The concentration of background dust used in mixtures was 10–100 times the typical mass of dust expected from a 12 h aerosol sample. This mass of background represents a worst-case scenario, one in which the atmospheric dust concentration is approaching the maximum concentration for a temperate region. Bulk powders of NIST SRM 2709 and counted batches of *B. subtilis* were completely digested and aliquots of these stock

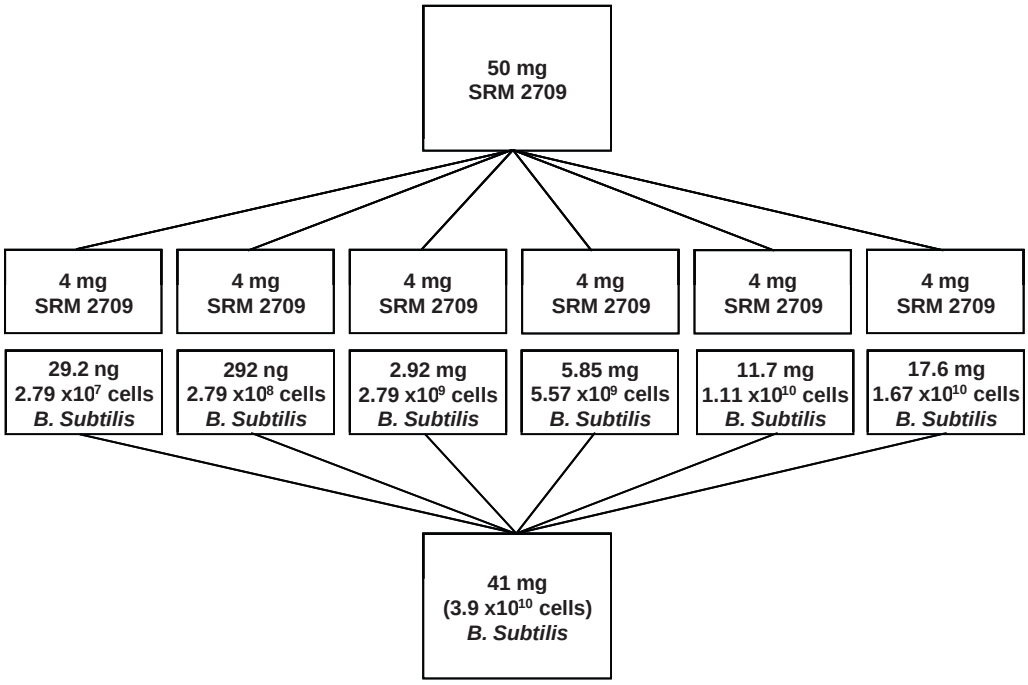

Fig. 1. The experimental design used to simulate the presence of *B. subtilis* at the ng-mg level in the presence of 4 mg of background dust is pictured here. A counted batch of the simulant and NIST SRM 2709 were dissolved separately and subsequently mixed in different proportions. This mixing approach is the most accurate method because of the uncertainty associated with accurately weighing sub-milligram sized samples of the simulant. The recoveries of the inorganic elemental fingerprint for both the SRM and the simulant are routinely near 100%.

solutions were then combined to simulate the presence of a biowarfare agent at low concentrations in the presence of background dust (Fig. 1). The justification for mixing digested solutions rather than powders is based on our ability to accurately determine the mass and therefore the number of bacterial cells below ~1 mg. The weighing error on small samples prohibits the direct mixing of powdered samples. Mixing solutions of digested powders eliminates this weighing error and allows for more accurate mixing proportions.

2.6. Selection of fingerprint components

The elements used for the inorganic fingerprint were based on the approach described by Gikunju et al. (2004)[1]. This approach selects elements based on their abundance in the bacterial sample and ease of measurement by ICP-MS. In this investigation elements that met these criteria also needed to be present in NIST SRM 2709 with published certified values. Based on these requirements, the following elements were used as components of the inorganic fingerprint: Mg, V, Cr, Mn, Co, Ni, Sr, Ba and Pb.

3. Results and discussion

The concentration (mg/kg) for each element selected as a fingerprint component, cell counts and simulated dust mass are presented in Table 2 for NIST SRM 2709, the bulk counted batch of *B. subtilis* as well as all bacteria:dust mixtures. The %RSD for all elements in each sample measured is typically better than 5% and recoveries for standard used to monitor external reproducibility are between 85% and 105%. In Fig. 2 the fingerprint for the bulk counted batch of bacteria and the NIST SRM 2709 sample are plotted on a log scale. The concentration of fingerprint components in SRM 2709 are all at least a factor of 3 greater than the concentrations in the bulk counted bacterial sample, making the bacterial fingerprint easy to distinguish from SRM 2709.

In Fig. 3 the fingerprints for the mixtures are plotted with the bulk bacteria and SRM 2709. In the presence of 4 mg of SRM 2709 only the mixtures with greater than 2.79×10^9 cells are easily discernable. While some components of the fingerprint are more sensitive than others, based on a principal components analysis, all elements in the mixed sample are significantly different from the bulk dust at the 2.79×10^9 level and above. This is approximately an order of magnitude above the minimum calculated sensitivity required to detect a toxic dose of inhalation anthrax. However, the dust levels in the mixtures analyzed are at ~4 mg which is at least an order of magnitude greater than that of a typical 12 h aerosol (i.e., 10–100 s of μg). A simple mixing model can be used to evaluate the sensitivity of the fingerprint approach at more typical dust levels.

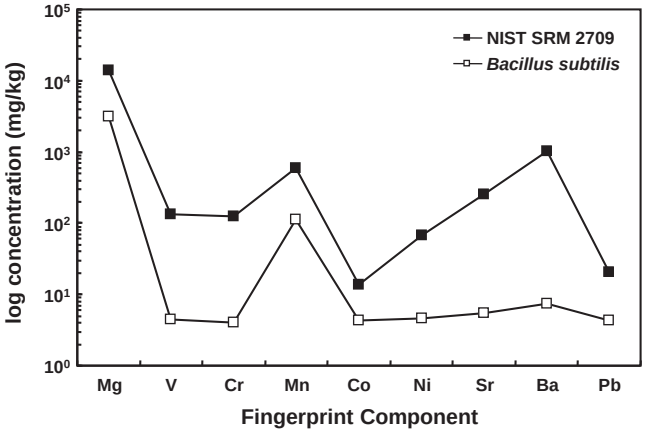

Fig. 2. This is a graph of the elemental fingerprint of *B. subtilis* (white box) and NIST SRM 2709 (black box) and experimental mixtures of these components (black). The recoveries of the inorganic elemental fingerprint for both the SRM and the simulant are routinely near 100%.

Table 2
Elemental fingerprint data (mg/kg) for NIST SRM, stock bacteria and all dust: bacteria mixtures. All values are better than ±5% RSD.

Sample ID	Mg	V	Cr	Mn	Co	Ni	Sr	Ba	Pb	*B. subtilis*	Cells
NIST SRM 2709	14160	126	115	544	13	62	248	1090	16		
B. subtilis stock	3103	4.4	4.1	111.8	4.3	4.7	5.5	7.3	4.4	3.90E+10	
Mix 1	15872	150.6	142.7	683.6	13.1	64.2	305.1	1254.6	24.9	2.79E+07	
Mix 2	15130	140.9	134.7	656.4	12.4	59.3	291.7	1194.1	23.5	2.79E+08	
Mix 3	10556	86.7	83.5	451.7	7.4	36.0	180.8	737.3	14.7	2.79E+09	
Mix 4	8568	60.9	59.1	360.0	5.6	25.5	128.1	522.3	6.8	5.57E+09	
Mix 5	6717	38.4	37.5	271.8	3.6	16.3	82.2	331.3	4.5	1.11E+10	
Mix 6	5699	27.3	27.3	233.7	2.6	12.0	61.0	240.4	3.4	1.67E+10	

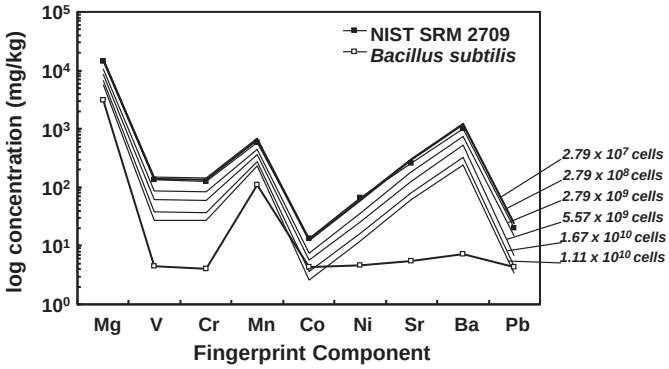

Fig. 3. This is a graph of the elemental fingerprint of *B. subtilis* (white box), NIST SRM 2709 (black box) and experimental mixtures of these components (black). These results clearly show that the limit of detection for *B. subtilis* in the presence of 4 mg of dust is approaching 2.79×10^9 cells (2.92 mg of stimulant). Lesser amounts of stimulant are not easily distinguished from the background SRM fingerprint.

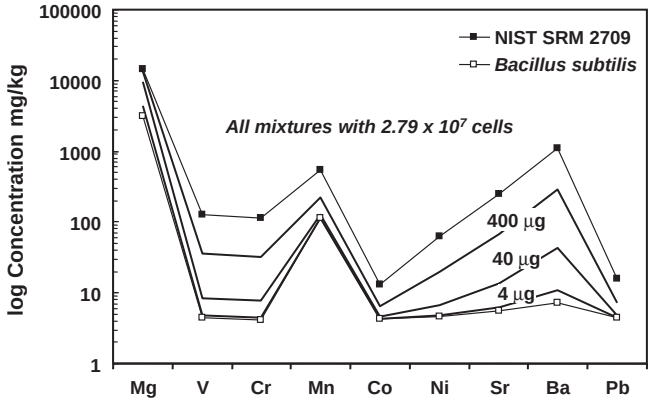

Fig. 4. The chemical fingerprints plotted here represent the results of a model mixing a sample of *B. subtilis* (white box) which contains 2.79×10^7 cells (29.2 ng of stimulant) and different amounts of NIST SRM 2709 (black box) more typical of aerosol samples. Based on this model the detection limit for the simulant in the presence of a 100 s of mg of background dust is below toxic effect threshold for *B. anthracis*.

In Fig. 4, a mixing model is presented that simulates a mixture between 2.79×10^7 cells of *B. subtilis* and the NIST SRM 2709 at levels lower than those measured. The physical mixture experimental results demonstrate the potential of the inorganic fingerprint approach but are limited by the need for a minimum sample volume (~5 mL) for analysis by ICP-MS. The model in Fig. 4 suggests the limit of detection for the inorganic fingerprint is well below the minimum toxic inhalation threshold for *B. anthracis* of 4.32×10^7 spores mixed with a background aerosol mass of between 4 and 400 μg. The values for all components, with the exception of Mg in the 400 μg mixture, of the inorganic fingerprint

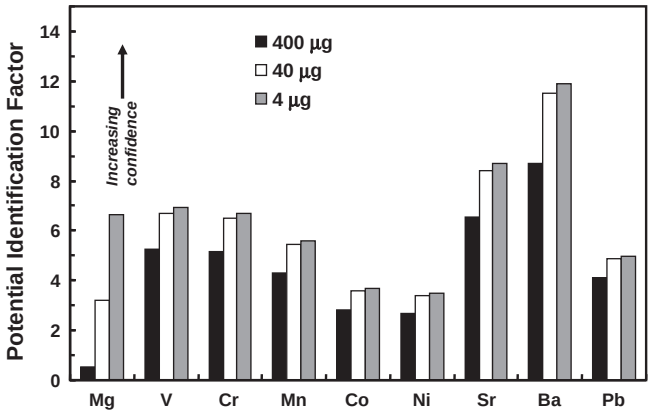

Fig. 5. The elemental fingerprint of the dust:bacteria mixture as compared to the composition of the dust stimulant (±95% confidence interval) and is plotted as the Potential Identification Factor for each model plotted in Fig. 4. In all simulated mixtures all elements, with the exception of Mg in the 400 mg dust mixture, are depleted from the original dust composition by at least a factor of outside the 95% confidence interval on the dust measurements.

are outside the 95% confidence interval determined for SRM 2709 (Fig. 5).

4. Conclusions

This study presents results that illustrate the potential of the inorganic fingerprint approach for the detection of a biological agent in the presence of a complex matrix. The ability to conclusively detect the presence of a non-background bacterial spore below the toxic effect threshold provides data to support further research and development of this approach. This technology could also be used in conjunction with other detection methods, such as laser scattering and UV-fluorescence, to create an integrated detection device capable of detecting the presence of biological particles and characterizing their chemical signature in real time. This will provide the capability to detect and identify biological weapons agents, while at the same time provide information on their source.

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