FAST VIABILITY ASSESSMENT OF *CLOSTRIDIUM* SPORES—SURVIVAL IN EXTREME ENVIRONMENTS

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Wanwan Yang

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"Everything should be made as simple as possible, but not simpler."

Albert Einstein

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Abstract

Bacterial endospores are formed in genera such as *Bacillus* and *Clostridium* in response to adverse environmental changes. Endospores have remarkable resistance to various extreme conditions and can remain dormant for extended periods of time. *Clostridium* spores are of particular interest due to their significant importance in several industries, such as food processing, wastewater treatment, pharmaceuticals, and health care. They are also the ideal candidates to study *Panspermia* and potential extraterrestrial life. However, to date, most endospore research has been conducted on *Bacillus*, and study of the anaerobic spore former, *Clostridium*, is not adequate.

In this study, we have developed a general protocol to produce and purify *Clostridium* spores. Spectroscopy and microscopy based Endospore Viability Assay (Spectro EVA and Micro EVA) were developed and validated to assess the viability of *Clostridium* spores. Germinability was used as an indicator for spore viability. The basic principle of the two EVAs is to measure the release of a unique biomarker, dipicolinic acid (DPA), *via* germination as a proxy for endospore viability. In particular, a luminescence time-gated microscopy technique (Micro EVA) has been developed to enumerate germinable *Clostridium* endospores within an hour. Micro EVA is based on energy transfer from DPA to terbium ions doped in a solid matrix upon UV excitation. The distinctive emission and millisecond lifetime enables time-resolved imaging to achieve single endospore sensitivity. Comparing to traditional CFU cultivation, EVA probes the early stage of germination, resulting in a much faster detection rate (within 60 minutes) than CFU measurement (more than 3 days incubation). Micro EVA has also been successfully

applied to quantify *Clostridium* spores in an extreme cold biosphere, Greenland ice core, and a hyper-arid biosphere, Atacama Desert, two Mars analogs on earth.

The development of EVA provides a faster way to assess viability of *Clostridium* spores, which has significant importance in various industries. It also enables the determination of the limit and longevity of life, and provides insight on the search of extinct or extant life on Mars and other celestial bodies.

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CHAPTER 1: INTRODUCTION

1.1 OVERVIEW OF SPORE FORMERS

Bacterial spores (i.e., endospores), first discovered by various research groups independently in 1876 are almost exclusively found in gram positive bacteria [1-3]. The formation of endospore is one of the defining traits of genera such as *Bacillus*, *Clostridium*, *Thermoactinomyces*, *Sporolactobacillus*, and *Sporosarcina*. *Desulfotomaculum* and *Sporomusa* are two types of spore formers that have gram negative staining but have a gram positive type cell wall and share 16s rRNA sequences with *Clostridium* species. Currently, the only well-known gram negative spore former is *Sporohalobacter* [3].

Endospores are formed by intracellular division within the cytoplasm of a mother cell. Spore-forming bacteria initiate sporulation in response to adverse environmental changes, such as nutrient limitation. After being through a sequential sporulation stages, the mature spores are released from the mother cells [3, 4]. The resultant endospore form exhibits incredible longevity, tenacity and persistence facing extreme environmental stresses (i.e. extremes of temperature and pH, high/low pressure, desiccation, UV radiation and attack by a wide variety of oxidants) that would kill growing cells of nearly all other bacterial species [5]. Several published reports have claimed the isolation of viable spore-forming bacteria from the gut of a bee trapped in Dominican amber about 25~40 million years ago [6], and even more spectacular, the recovery of spores from 250-

million-year-old halite crystals [7]. A six-year space study also showed that spores of *Bacillus subtilis* survived after exposure to space environments such as high vacuum and radiation [8].

The unique structure and chemical composition of endospore play major roles in its extreme resistance to various stresses. Figure 1.1 shows the internal structure of a typical bacterial spore. Starting from outside and proceeding inward, the spore includes exosporium, coat, cortex, inner membrane and central core. The exosporium is composed of proteins with unknown functions. The coat is a complex structure with multiple layers, which is important in spore resistance to some chemicals, such as exogenous lytic enzymes that can degrade spore cortex. However, the coat has little or no role in spore resistance to heat, radiation and some other chemicals. The cortex is made up of peptidoglycan, which is essential for spore formation and the reduction of water content in spore core. The inner spore membrane serves as a strong permeability barrier that plays an important role in spore resistance to many chemicals, especially to those that cross the membrane to damage DNA in the core. The spore core is an analogue of the protoplast of the growing cell, which contains most spore enzymes, DNA, ribosomes and tRNAs. One unique feature of endospore is the low water content. Unlike the protoplast of a growing cell, in which water comprises 75~80% of its wet weight, endospore only contains 27~55% water as its wet weight. The amount of free water in spore core is extremely low, which restricts the macromolecular movement. The low water content affects the refractive index of spore, which leads to the phase bright appearance of spores under phase contrast microscope. Low water content is also the major factor that accounts

for the enzymatic dormancy of endospore and its resistance to wet heat. Another unique molecule in spore core is pyridine-2,6-dicarboxylic acid (dipicolinic acid, DPA), which plays significant role in the UV photochemistry of spore DPA and its resistance to UV radiations [9-11].

The life cycle of a spore-forming bacterium comprises of three processes: vegetative growth, sporulation and germination (Figure 1.2) [4]. Vegetative growth occurs when nutrients are available and is characterized by cells growing logarithmically by symmetric fission. Cells adjust growth when nutrient levels fall. If nutrients become too scarce (i.e., the onset of starvation), cells cease growing exponentially and enter stationary phase. During the transition to stationary phase, cells initiate various responses to adapt to the adverse conditions and increase competitiveness against other species, such as chemotaxis and motility, synthesis of antibiotics and toxin, expression of transport systems, induction of catabolic pathways, and activation of the genetic competence cascade. The function of these responses is to help the cell to reach, to liberate (by killing neighboring cells), to take up and to metabolize potential secondary sources of nutrients. The cells will commit to sporulation when all these efforts fail. Once a cell commits to sporulate, it must complete the process and become a dormant spore [3]. Albeit its resistance to environmental insult and its metabolic dormancy, endospore is able to recover its metabolism as an active growing cell in response to favorable environmental conditions, such as presence of water, nutrient and germinants. As an endospore proceeds through germination towards cell division, there are various stages, including (1) spore activation; (2) stage I germination, during which water partially

rehydrates the spore core and DPA is released; (3) stage II germination, during which cortex hydrolysis occurs and metabolism resumes; and (4) outgrowth, during which cell division occurs [4, 12-14]. Each of the stage can be observed by different environmental technique described below.

Endospores are of significant importance to scientists due to their ubiquity in the natural environment and their resistance to various stresses. *Bacillus* and *Clostridium* contain the causative species for anthrax, tetanus, botulism, and gas gangrene [15]. Since spores can survive various sterilization processes, spore formers are causative agents for various food-borne diseases. *Bacillus cereus* is a common aerobic food-borne pathogen [16], and *Clostridium botulinum* and *Clostridium perfringens* are anaerobic food-borne pathogens commonly associated with food poisoning from canned foods [15]. Similarly, the survival of pathogenic spore-forming bacteria under various disinfectant treatments is one of the main causes of hospital acquired infections [17, 18]. The most virulent species is *B. anthracis,* which has been released as bioweapon [19, 20]. Despite the ominous toxicity, spore formers have some industry importance as well. The proteases secreted by *B. subtilis* are a common additive to laundry detergents; the α-amylases and glucose isomerase secreted by *B. amyloliquefaciens*, *B. licheniformis*, and *B. stearothermophilus* are very useful in converting starch to corn syrup and dextrose; *B. thuringiensis* are natural insecticides; the xylanase of *B. stearothermophilus* is widely used in the paper pulp industry; and *B. subtilis* natto is the agent that produces a fermented soy product commonly eaten for breakfast in Japan [3]. *C. acetobutylicum* has been used to produce significant amount of acetone/butanol/ethanol by large scale industrial fermentation. *C.*

thermocellum has been used in cellulose degradation. Endospore formers are also the producers for important antibiotics, such as peptides bacitracin, fengycin, polymyxin, gramicidin and tyrocidine [3].

Bacillus and *Clostridium* spores are also of great interests in sterilization control. Spores of these two genera have been used to monitor the sterility processes in various industries. Residual endospores on spacecraft surfaces after disinfection post potential jeopardy to the samples collected from other planets and may also give rise to growth of earth microorganism on these planets. Understanding properties of endospores aids in NASA's objective in planetary protection and preventing forward contamination. Owing to its high resistance to various environmental insults, bacterial spores can be recovered from almost all extreme environments on Earth. Study of the viable endospores entombed in polar ices, suspended in frozen lakes and embedded in permafrost also provides insights in longevity of life.

1.2 ANAEROBIC SPORE FORMERS (*CLOSTRIDIUM)*

By far the greatest amount of information is available for aerobic spores-formers, *Bacillus* species. There has been little detailed work conducted to study the sporulation and germination of *Clostridium*. Unlike *Bacillus*, *Clostridia* are anaerobic, which will not grow or sporulate under aerobic conditions. The vegetative cells are likely killed by exposure to O_2, but their spores can survive long periods of exposure to oxygen [21]. The sporulation processes is similar in these two genera, however, the initiation of this event is different. It has been well known that sporulation in *Bacillus* is triggered by nutrient

limitation; however, what triggers the sporulation in *Clostridium* is unknown. In this thesis, I will focus my study on *Clostridium* species.

The genus *Clostridium* was first proposed by A. Prazmowski in 1880, and since then more than 100 bacterial species have been assigned to it. To be classified as a *Clostridium* species, a microorganism has to meet four criteria: (1) it must be able to form endospores; (2) it must obligatorily rely on an anaerobic energy metabolism, while the anaerobic requirement for some pathogenic *Clostridium* species is less strict; (3) it must be unable to carry out a dissimilatory sulfate reduction; and (4) it must have a Gram positive type cell wall. Clostridial cells are straight or curved rods, 0.3~1.6 × 1~14 μm, with the exception of *C. coccoides*, which is coccal to rod shape. Cells are usually motile with peritrichous flagellation [22].

The genus is very heterogeneous, with the GC content of the DNA ranging from 21 to 54 mol%. *Clostridium* includes psychrophilic, mesophilic and thermophilic species. The major role of this genus in nature is to degrade organic compounds to acids, alcohols, carbon dioxide, and hydrogen. A butyric acid smell is frequently associated with *Clostridium* species. Some species are moderately aerotolerant (*C. aerotolerans*, *C. carnis*, *C. durum*, *C. histolyticum*, and *C. tertium*), while others, such as *C. aminovalericum* are extremely fastidious [22]. Although some species tolerate oxygen and are even able to grow under air, sporulation only occurs under anaerobic conditions, which is one of the major features that distinguish *Clostridium* from *Bacillus*.

Due to the ubiquitous distribution of *Clostridium* species in natural environment, the substrate spectrum of the whole genus is extremely broad and covers a wide range of naturally occurring compounds. On the basis of their preferred substrates, the genus can be divided into four different nutritional groups: (1) Saccharolytic clostridia, which are usually nonpathogenic, and able to grown on carbohydrates such as xylose, mannitol, glucose, fructose, lactose, and raffinose. This group includes starch-utilizing species (e.g., *C. butyricum*), cellulose-utilizing species (e.g., *C. cellobioparum*), pectin-utilizing species (e.g., *C. felsineum*), and chitin-utilizing species (i.e., *C. sporogenes*); (2) Proteolytic clostridia, which are able to excrete proteases and digest proteins. An unique feature of this group is the ability to ferment amino acids and form corresponding branched-chain fatty acids. Several species of this group are highly pathogenic, such as *C. botulinum* and *C. tetani*; (3) Proteolytic and saccharolytic clostridia, in which most species are pathogenic (e.g., *C. perfringens* and *C. sordellii*), with the exception of *C. oceanicum*; (4) Specialists, which are neither proteolytic nor saccharolytic. Microorganisms in this group are specialized on utilizing one or a few substrates. For example, *C. acidiurici* and *C. purinolyticum* grow on purines such as uric acid and adenine but not on sugars or amino acids. *C. kluyveri* ferments only on ethanol, acetate and bicarbonate to butyrate, caproate and molecular hydrogen. *C. propionicum* ferments only on threonine and three-carbon compounds such as alanine, lactate, acrylate, serine and cysteine. *C. cochlearium* degrades only glutamate, glutamine, and histidine [22].

The study of *Clostridium* becomes more and more popular due to its importance in various areas, such as the food industry, wastewater treatment, medical industry and

astrobiology. Spore of *Clostridium* species may survive various pasteurized processes, which causes spoilage in food stuffs later on. *C. perfringens* causes human gas gangrene and two very different foodborne diseases: a relatively mild type A diarrhea, and a very serious but rare type C human necrotic enteritis. It also causes many animal diseases such as enterotoxaemia in mammals and necrotic enteritis in birds. The production of one or more toxins by *C. perfringens* is the cause of these diseases. Botulism, a rare but extremely dangerous disease with high mortality, is caused by a neurotoxin produced by *C. botulinum* and related species (*C. baratii* and *C. butyricum*). *C. tyrobutyricum* causes spoilage of cheese by gas formation and off-flavor development. The butyric acid clostridia, *C. butyricum* and *C. pasteurianum* may spoil canned fruits occasionally with a pH as low as 3.7. Psychrotrophic clostridia (e.g., *C. gasigenes* and *C. estertheticum*) survive and grow at low temperatures, which cause spoilage of chilled vacuum-packed meat by forming gas and developing off-odors. These psychrotrophic clostridia can also multiply and spoil fresh meat, milk and potatoes maintained at low temperatures [23]. The study of *Clostridium* spore inactivation is crucial to develop the standard of hygiene in various control procedures in food industry.

Sulfite-reducing clostridia are exclusively fecal in origin [24], and their spores are very resistant and can survive in water much longer than coliforms or streptococci. Spores of sulfite-reducing clostridia (SSRC) are also resistant to disinfection and therefore not ready to reduce by various treatments. So it may not be an ideal candidate to assess water treatment efficiency; however, they are of use in assessing the efficiency of filtration and the susceptibility of water sources to intermittent pollutions [24]. Spores can survive very

long period of time, and in such cases spores may be detected long after a pollution incident giving rise to false alarm. This is one of the reasons that they are not used as fecal indicators in the USA. However, SSRC have been considered as an indicator for the presence of pathogenic microorganisms in drinking water by European Community legislators [25].

Clostridium spores have important application in the medical and health industry. *C. difficile* and *C. tetani* are etiological agents that cause hospital acquired infections [17, 18]. Despite the toxicity of some *Clostridium* species, non-pathogenic clostridia have been used to target tumors with gene therapy in cancer research. The majority of solid tumors contain regions of low oxygen or dead tissue, where the traditional radiotherapy and chemotherapy are ineffective. The anaerobic environment encourages growth of *Clostridium* species, and their spores survive oxygen rich environment. If clostridial spores are injected into an animal with cancer they spread throughout the body, only spores that reach an oxygen starved area of a tumor will germinate, multiply and become active. Engineered clostridia have been shown to successfully target tumors and deliver therapeutic gene, and safe and effective anti-tumor results are reached [26].

Clostridia also produce interesting fermentation products and secrete useful enzymes and proteins. *C. acetobutylicum* was used for approximately 30 years on a large scale for acetone/butanol production. Acetogenic clostridia (e.g., *C. thermoaceticum*, *C. thermoautotrophicum*, and *C. formicoaceticum*) have been studied as potential producers for calcium-magnesium acetate as deicer. *C. thermohydrosulfuricum*, *C. thermocellum*

and *C. saccharolyticum* have also been investigated to produce ethanol. Some *Clostridium* species are good sources of stable enzymes. Hyun and Zeikus reported the simultaneous and enhanced production of thermostable amylases and ethanol from starch by cocultures of *C. thermosulfurogenes* and *C. thermohydrosulfuricum*. *C. thermosaccharolyticum* has also been reported to produce pullulanase, an important enzyme in sugar syrups production. *C. thermocellum* is the most abundant cellulolytic species, which has potential to produce large amount of cellulases, an enzyme responsible for the conversion of cellulosic biomass to useful chemical products (e.g., ethanol) [27].

Due to its anaerobic property and high resistant to various extreme conditions, *Clostridium* spores also have potential applications in astrobiology. *Clostridium* spores will be the perfect model microorganism to study the potential growth of earth microorganisms on Mars, considering a highly anaerobic Mars atmosphere (with 95.3% CO_2). They are also the ideal microorganism to test the Panspermia hypothesis that life is transported from one planet to another and endospores are one of the microorganisms that most likely survive an interplanetary journey [28, 29]. In planetary protection, *Clostridium* spores can serve as an indicator to monitor the sterility of spacecraft before launching, which has significant application in preventing forward contamination from Earth to other planets.

1.3 OVERVIEW OF SPORE DETECTION METHODS

Currently, the standard method for quantifying endospores is to enumerate colony forming units (CFU) after heat-shock treatment and several days of incubation. The

advantage of CFU count is that it gives actual cell number and diversity of the sample under analysis. However, this method is very time consuming, and moreover, tedious anaerobic techniques are required for *Clostridium* sores. Furthermore, when analyzing environmental samples, the culturability may be as low as 0.1% or 0.01%, known as the viable but not culturable (VBNC) phenomenon. So, using cultivation-based methods tends to underestimate the total spore counts.

Recently, direct polymerase chain reaction (PCR) has been applied to detect spores of *B. anthracis* [30] and *C. tyrobutyricum* [31], which require disruption of spores by glass or zirconia beads or microwaving to release DNA from spores. Quantitative PCR methods are shown to be extremely sensitive, with detection limits of fewer than 10 cells per mL, and analysis times of approximately 3 h. However, the PCR technique requires that the bacteria and spores be disrupted to make the endogenous DNA available for amplification. Bacterial spores are particularly difficult to process, as their nucleic acid is encased in a very resistant shell. Therefore, direct PCR is a very expensive and labor intensive detection method.

Bacterial spores can also be detected with specificity using fluorescent-conjugated polyclonal antibodies directed towards the spore coat, based on the interaction between antibodies and bacterial spore cell surface antigens [32-34]. Cardosi et al. described a sensitive two-site, enzyme-linked immunoassay for *C. perfringens* phospholipase C (atoxin). The approach incorporated an electrochemical detection step based on thin-layer hydrodynamic voltammetry coupled with fast liquid chromatography with

electrochemical analysis (LCEC) [35]. The advantage of this technique is its specificity to the particular microorganisms. However, its limitations are due to the requirement of intensive labor and expensive reagents.

We propose a new method for spore detection, which targets the unique biomarker of bacterial spores, DPA. DPA can be release by induced germination (simply adding germinant to spore suspensions) or autoclaving spores. DPA is released in the early stage of germination, which makes a rapid detection method possible. The new method also requires minimal labor work, and most germinants are inexpensive amino acids.

Terbium^{3+}-DPA (Tb^{3+}-DPA) luminescence assay forms the basis of spore detection method developed in my thesis. Tb^{3+}, a lanthanide ion, is a unique fluorescence metal with decay time as milliseconds [36-38]. When Tb^{3+} absorbs a photon or is otherwise supplied with a sufficient quantum of energy, it reaches an electronically excited state. The excited state is not stable, and energy loses *via* radiative transition, with the emission of a photon as the electron transfer back into its lower energy orbital. This is known as fluorescence. The emission of Tb results from transitions involving 4*f* orbitals, which are forbidden transitions with very low absorption coefficients. That is why the emissive rates are low for Tb, resulting in long lifetimes. Also, due to its weak absorption, Tb is usually not directly excited but rather excited through chelated organic ligands by energy transfer [37].

When absorbing photons, optimally in the UV range, DPA is promoted from its ground state to a vibrationally excited singlet state. This excited singlet can lose its excess energy by both radiative and nonradiative decay. DPA has a matched electronic transition levels with Tb^{3+}, which makes an efficient intersystem crossing possible. In addition, the energy difference is well above the $^5D_4$ state of Tb^{3+}, so the back transfer into DPA triplet state is not possible. When chelating with Tb^{3+}, energy is transferred from the lowest-lying DPA triplet excited state to the emissive $^5D_4$ state of Tb^{3+}. In this case, DPA acts as a light harvesting antenna to receive UV excitation and then transfers the energy to Tb^{3+}, and Tb^{3+} will emit photons at its characteristic wavelengths as it returns back to its lower energy stages. The original Tb^{3+} excitation spectrum (λ_{ex} = 270 nm) will be changed to a characteristic dual-peak spectrum of DPA (λ_{ex} = 273, 279 nm) upon complexation with DPA. Because the energy gap between the Tb^{3+} ground state and emissive states is very large (20,500 cm^{-1}), the luminescence enhancement is very significant (>20,000 times) [37], according the energy gap law. Figure 1.3 shows the Jablonski diagram of the absorption-energy transfer-emission (AETE) mechanism from DPA to Tb^{3+}.

Lanthanide ion fluorescence is chosen to detect DPA due to two of their useful analytical properties: the fluorescence lifetime of lanthanide ions are as long as several milliseconds and the ion fluorescence are substantially enhanced by energy transfer from a chelated DPA. Tb is the best lanthanide for DPA detection due to its brighter fluorescence, longer fluorescence lifetime and higher chelating enhancement ratio [36-38]. Background and autofluorescence signals usually decay rapidly with lifetimes on the timescale of 1~100 nanoseconds [37]. Terbium dipicolinate has a lifetime in the range of milliseconds.

Therefore, when excited by UV, the emitted Tb^{3+}-DPA intensity can be observed without interference from autofluorescence and scattering light, resulting in a substantial increase in detection sensitivity.

1.4 OUTLINE OF THESIS

Clostridium species are ubiquitous, with significant industrial and scientific importance. However, due to the tedious anaerobic growth requirements and the lack of genetic tools for *Clostridium*, studies on this genus are inadequate. In my thesis, my goals are to develop and validate spore detection techniques for *Clostridium* spores, to distinguish them from their aerobic counterpart, and to apply these techniques to various environmental samples. My thesis work contributes to filling in the knowledge gap for anaerobic spore-forming microorganisms, providing useful tools for scientists to study *Clostridium* species in the food industry, wastewater treatment and pharmaceuticals, and assessing the distribution of *Clostridium* spores in various extreme ecosystems on Earth and the longevity of this toughest life form.

Chapter 2 describes a novel method to successfully sporulate various *Clostridium* species. The production of pure *Clostridium* endospore suspensions is indispensable for investigating their physiology, chemistry, and industrial applications. The biggest challenges for the production of pure *Clostridium* spore suspensions are strain degeneration and the unsynchronized growth of this genus. I have overcome these challenges and provided a general protocol for optimizing the production of pure, high

concentration *Clostridium* endospores. This protocol is used through all my thesis work to produce various pure *Clostridium* spore suspensions.

Chapter 3 describes the development and validation of a spectroscopy based method, Endospore Viability Assay (Spectro-EVA) to detect *Clostridium* spores in liquid samples. Spectro-EVA is based on the detection of a bacterial spore biomarker, DPA with Tb^{3+}-DPA luminescence. DPA is released either by germination and autoclaving, and the resulting DPA concentrations are correlated to the concentrations of germinable spores and total spores in a given sample. The ratio of germinable to total spores indicates the proportion of germinable spores in a given spore suspension. Specto-EVA has been applied to study the germination dynamics of *C. sporogenes* spores as a function of temperature in the range of 30 °C to 60 °C, and to study the difference between *Bacillus* and *Clostridium* spores responding to various germinants.

Chapter 4 details the development of a microscopy based, Tb^{3+}-DPA luminescence method to detect and quantify *Clostridium* spores on solid matrix, known as the microscopic endospore viability assay (Micro-EVA). Micro-EVA has been fully validated with cultivation and phase contrast microscopy on enumerating pure endospore suspension. Unlike Spectro-EVA described in Chapter 3, Micro-EVA is developed to quantify *Clostridium* spores from solid samples from various environments, and diluted liquid sample with minimal viable microorganisms. This detection method has also been applied to distinguish aerobic and anaerobic spores.

Chapter 5 describes the application of these two spore detection methods (Spectro-EVA and Micro-EVA) to detect and enumerate viable *Clostridium* spores from various extreme environment samples. This includes samples from Greenland ice core and Atacama Desert. The ice core samples include a transect of different depths extending from 600 to 110,000 years old. The Atacama samples include soils from subsurface of the most arid site, Yungay, and three depths at a mine pit. The goal is to assess the distribution and abundance of viable anaerobic *Clostridium* spores in various environments and to shed light on the longevity of this genus.

Chapter 6 concludes the thesis with various potential applications of these detection methods. With the importance of *Clostridium* in food and medical industry, EVAs will be able to find its applications in monitoring the food safety and evaluate the cleanness of various medical devices. Since EVAs target the early stage of spore germination, by coupling with other methods, such as monitoring optical density change, ATP methods, phase contrast microscopy and enumeration of CFUs, EVAs will provide insight on the different stages of *Clostridium* spores germination under various conditions.

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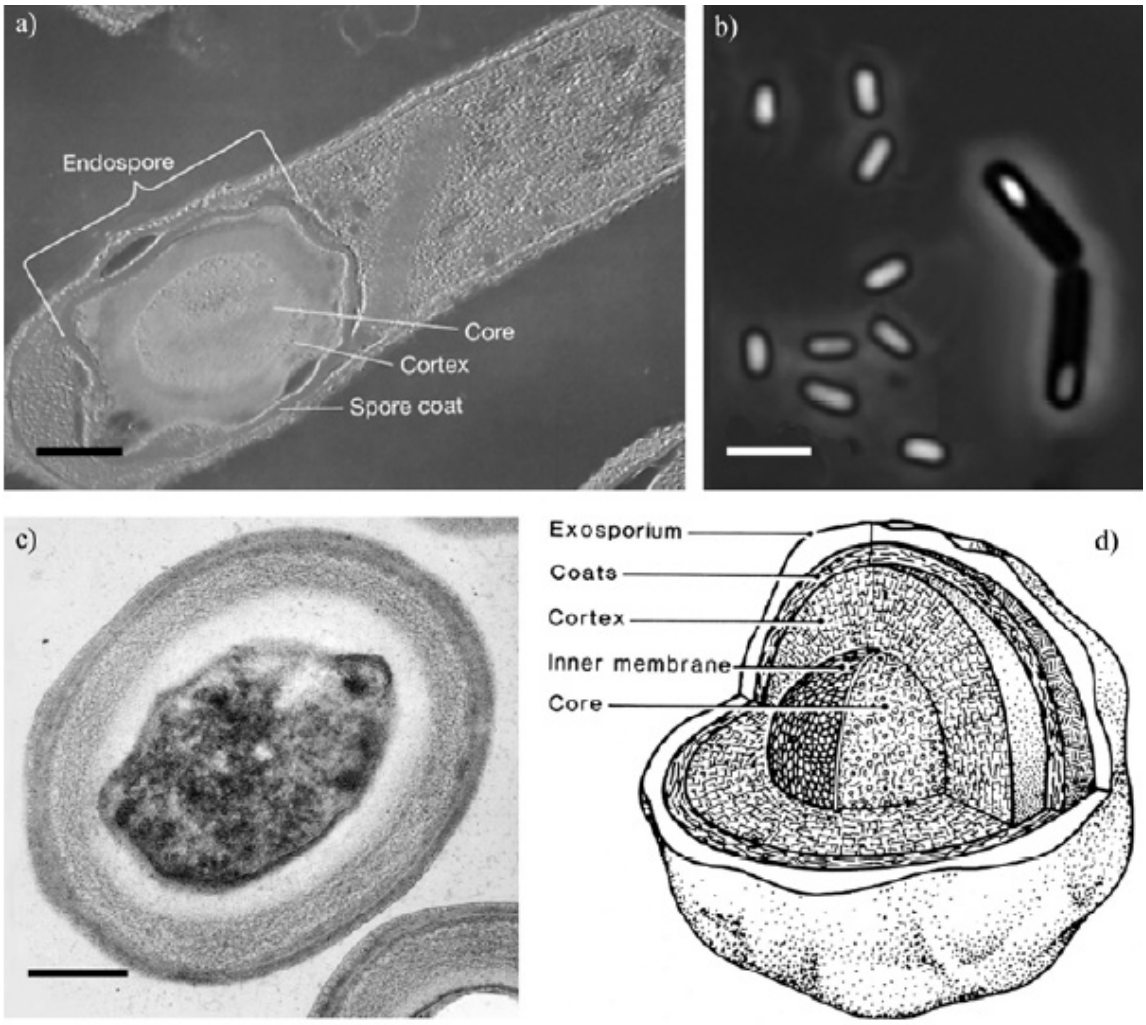


Figure 1.1 (a) Transmission electron micrograph of a bacterial spore embedded within a *Clostridium perfringens* vegetative cell. Scale bar = 0.3 μm. **(b)** Bacterial spores appear phase bright observed under a phase contrast microscope and are readily distinguished from the phase-dark rod-shaped vegetative mother cell. Scale bar = 2 μm. **(c)** Cross section of a TEM of an endospore of *Geobacillus stearothermophilus*. Scale bar = 0.2 micron. **(d)** Schematic representation of the internal structure of a typical bacterial spore, reproduced from Foster and Johnstone (1990).

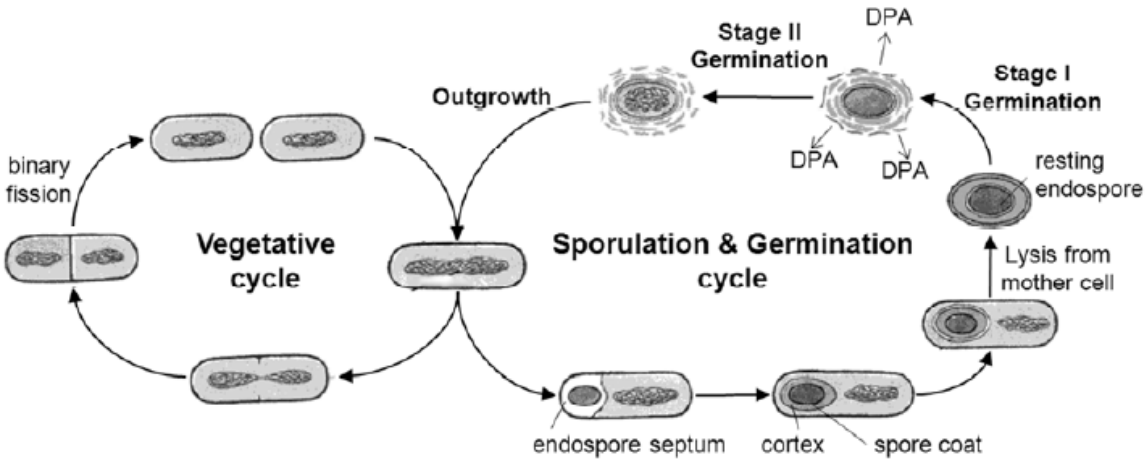
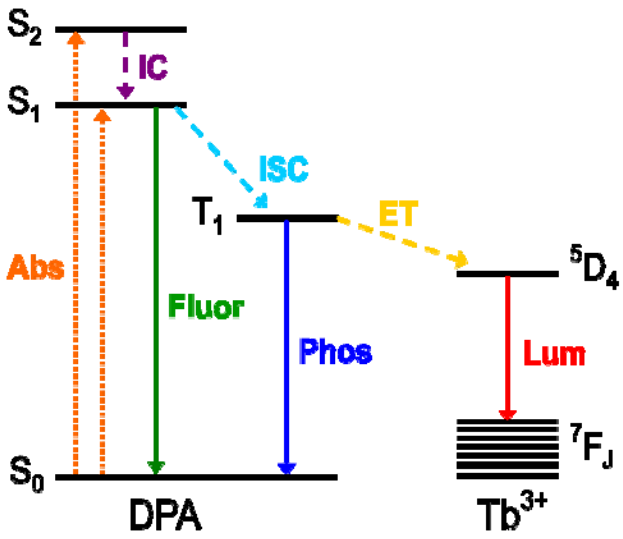
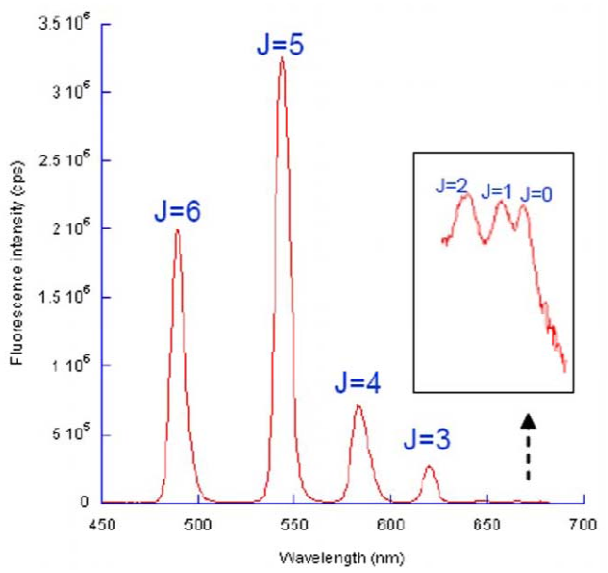


Figure 1.2 Three-stage life cycle of an endospore-forming bacteria: vegetative growth, sporulation and germination.



Abs	Absorbance
Fluor	Fluorescence
Phos	Phosphorescence
IC	Internal conversion
ISC	Intersystem crossing
ET	Energy transfer
Lum	Luminescence



J	wavelength (nm)	Property
6	480 – 505	Hypersensitive
5	535 – 555	Strongest optical activity
4	580 – 600	Hypersensitive
3	615 – 625	Strong optical activity
2	640 – 655	Weak: hypersensitive
1	660 – 670	Weak
0	675 – 680	Weak

Figure 1.3 Jablonski diagram of the absorption-energy transfer-emission (AETE) mechanism from DPA to Tb^{3+}. UV radiation is absorbed by the conjugated π-electron system of the DPA leading to a singlet excited state, which flows to the ligand triplet excited state and then the emittive level ($^5D_4$) of the Tb ion through intersystem crossing. Luminescence is observed through radiative decay from the excited state to the seven energy levels of the Tb^{3+} heptet ground state (^7F_J).

CHAPTER 2: PRODUCTION AND CHARACTERIZATION OF PURE *CLOSTRIDIUM* SPORE SUSPENSIONS*

2.1 ABSTRACT

As the study of anaerobic spore-forming clostridia becomes increasingly important due to their ubiquity in the natural environment and their importance in various industries, the production of pure *Clostridium* endospore suspensions is indispensable for investigating their physiology, chemistry, and industrial applications. In this chapter, we have developed two sporulation methods that yielded high concentrations of notably pure *Clostridium sporogenes* and *C. hungatei* spore suspensions (10 mL of 10^9 spores/mL with >99% purity each). Each method was derived by evaluating combinations of three sporulation conditions, including freeze drying of inocula, heat-shock treatment of cultures, and subsequent incubation at suboptimal temperatures that yielded the highest percentage of sporulation. Pure spore suspensions were characterized in terms of dipicolinic acid content, culturability, decimal reduction time (D)-value for heat inactivation (100 ˚C) and hydrophobicity. Our results show that while some *Clostridium* species produce a high percentage of spores with heat-shock treatment and suboptimal temperature incubation, other species require the additional step of freeze drying the inocula to achieve a high percentage of sporulation. The protocol we derived here optimizes the production of pure, high concentration *Clostridium* endospore suspensions, which are required for investigating species of medical and environmental importance.

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W.-W. Yang[1], E. N. Crow-Willard[2] and A. Ponce[1,2]
[1]California Institute of Technology, Pasadena, CA 91125
[2] Jet Propulsion Laboratory, Pasadena, CA 91101

Defining the conditions for optimal spore production also provides insight into the underlying mechanisms of *Clostridium* sporulation.

2.2 INTRODUCTION

Endospore-forming bacteria (e.g., *Bacillus* and *Clostridium*) initiate sporulation [1, 2] to survive periods of environmental extremes that are unfavorable for growth and readily kill vegetative cells [3]. The study of anaerobic spore-forming clostridia has become increasingly important due to their ubiquity in the natural environment [4-6] and their importance in medical and industrial applications, which has been thoroughly illustrated in Chapter 1 and can be briefly summarized as follow: *Clostridium botulinum* and *C. perfringens* are common food-poisoning agents that produce toxins which cause diseases such as botulism and human necrotic enteritis [7, 8]. In addition, *C. perfringens*, *C. difficile* and *C. tetani* are causative agents of gas gangrene, pseudomembranous colitis and tetanus [7, 9]. Some psychrotrophic clostridia are also responsible for the spoilage of chilled vacuum-packed meat [8]. *Clostridium* species have been used in industry for beneficial purposes. For example, *C. acetobutylicum* has been used to produce significant amounts of acetone/butanol/ethanol by large-scale industrial fermentation [10, 11]. Finally, *C. perfringens* has been used as an indicator of present fecal contamination as well as a conservative tracer for recent past fecal contamination events [12, 13], because it is present in large numbers in human and animal wastes.

The production of pure *Clostridium* endospore suspensions is indispensable for investigating their physiology, chemistry, and industrial applications. In contrast to aerobic *Bacillus* species, research of *Clostridium* species has been limited by the difficulty in producing pure endospore suspensions and tedious anaerobic growth requirements. The biggest challenge for the production of pure *Clostridium* spore suspensions is its typically unsynchronized growth habits. Most *Clostridium* cultures contain all possible cell forms: young vegetative cells, cells in various stages of sporulation, free spores, and germinated spores [14-16]. Past efforts to harvest clean spores from such a mixture have been unsuccessful [15, 17]. Another challenge to spore production is due to a process known as strain degeneration [18, 19], where loss of spore production occurs after repeated subculturing or during growth in continuous culture. Degeneration appears to be facilitated by excessive acidification of cultures during exponential growth, and by a global regulatory gene responsible for strain degeneration [19].

The first successful attempts to overcome these obstacles and increase synchronized sporulation were applied to *C. sporogenes* and *C. roseum* by increasing inoculum size (up to 10% v/v), heat-shock treatment of cultures, and multiple successive transfers of log phase culture before scaling up for spore production, resulting in up to 95% sporulation [14-16, 20]. However, these techniques did not apply universally to all *Clostridium* species. For example, Kihm *et al.* found that a zinc containing medium stimulated 70% sporulation of *C. botulinum* 113 B, although this stimulating effect was not pronounced with other *C. botulinum* strains [21]. Long *et al.* showed a high degree of variation

between strains of *C. acetobutylicum*, with respect to their ability to sporulate on various media, and was only able to obtain a maximum of 70% sporulation for one of the strains tested while the other strains had yields as low as 10% [22]. A similar conclusion was reached by other investigators in attempts to optimize the process of *C. perfringens* sporulation [23, 24], and their results also indicated strain-dependent effects on various sporulation media.

We have adapted and modified the most successful techniques previously developed and have demonstrated that synchronized growth and subsequent high endospore yields can be readily purified to a 99% pure endospore suspension for *C. sporogenes* and *C. hungatei*. Specifically, we examined the effects of freeze drying, suboptimal growth temperature and heat-shock treatment on the sporulation of *C. sporogenes* and *C. hungatei* and determined optimal sporulation conditions for these two species. Finally, we characterized these pure spore suspensions in terms of the dipicolinic acid (DPA) content, decimal reduction time (D)-value for heat inactivation (100 ˚C), and hydrophobicity of spores.

2.3 MATERIALA AND METHODS

Materials

Deionized water (18.2 MΩ/cm) was obtained from an ultrafilter system (Water Pro PS, LabConco, Kansas City, MO). Terbium (**III**) chloride hexahydrate-(99.999%), dipicolinic acid (99%) (2,6-pyridinedicarboxylic acid, DPA) were obtained from Aldrich

(Milwaukee, WI). Reinforced clostridial medium (RCM), trypticase and yeast extract were purchased from BD Diagnostics (Franklin Lakes, NJ). Ammonium sulfate was purchased from Mallinckrodt (Carlsbad, CA). *Clostridium sporogenes* (ATCC No.7955) and *Clostridium hungatei* (ATCC No.700212) [25] were purchased from American Type Culture Collection (ATCC) (Manassas, VA) as freeze-dried pellets.

Phase contrast microscopic enumeration to determine spore concentrations

Aliquots (5 μL) of spore suspension were placed in a Petroff-Hausser counting chamber (Model 3900, Hausser Scientific, Horsham, PA,), and spores were observed at 400× magnification using a phase contrast microscope (Nikon Eclipse 80i, AG Heinze Co, Lake Forest, CA) mounted with a digital camera (Nikon Digital Sight DS-5M). The smallest squares in the counting chamber are 0.05 mm × 0.05 mm, of which 80 were analyzed to obtain the average spore count per square. To obtain statistically significant counts, the spore concentration was held above 10^7 spores/mL, which resulted in at least 10 cells per sixteen squares.

Endospore production

C. sporogenes and *C. hungatei* were revived from frozen, dry pellets in a small volume (5~6 mL) of growth media at the optimal growth temperatures of 37 °C and 30 °C, respectively. The growth media for *C. sporogenes* and *C. hungatei* was reinforced clostridial medium (RCM) and ATCC 2135 broth (GS-2CB medium), respectively. Incubation commenced under strict anaerobic conditions with 100% N_2 headspace. For

sporulation of *C. sporogenes*, a 10% inoculum from a growth culture was transferred to 75 mL of sporulation medium {3% trypticase, 1% peptone and 1% $(NH_4)_2SO_4$ [26]}. After heat-shock treatment at 80 ºC for 15 min, it was incubated at the suboptimal temperature of 30 ºC (7 °C below optimal) while shaking at 180 rpm. *C. hungatei* was sporulated in the same manner as *sporogenes*, however, its sporulation medium was the same as its growth medium and it was grown at the suboptimal temperature of 23 °C. To facilitate sporulation, a frozen 15% glycerol stock of *C. hungatei* was freeze-dried while still in the cryovial in a benchtop freeze dry system (Freezone 4.5, Labconco, Kansas City, MO). This dry pellet was then revived in 10 mL growth medium. A similar procedure was applied to sporulate an environmental isolate, *Clostridum* G5A-1, a strain isolated in our laboratory from Greenland Ice Sheet Project 2 (GISP-2) at 1566 m, an ice core dated to be 10,000 years old. The sporulation temperature for this new isolate was 23 °C, since the optimal growth temperature of this strain has not been determined yet. The cultures were monitored by phase contrast microscopy for spore production on a daily basis using the method described above.

Endospore purification

After incubation in sporulation media at suboptimal temperature for 5 to 6 days when most spores were released from the mother cells and total sporulation had reached above 90%, spores were harvested and cleaned following a revised procedure adapted from methods for *Bacillus* and *Clostridium* spores [1, 14, 16]. Our method is summarized as follows. Endospores were harvested and purified by centrifugation. The spore suspension (80 mL) was centrifuged at 12,850 g for 10 min at 4ºC. The endospores were

washed once by resuspending the pellet in 20 mL (1/4 volume) of deionized water followed by repeat centrifugation. The spore pellet was then resuspended in 1/4 volume of 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, pH 7.4) containing 500 µg/mL lysozyme (Sigma, St. Louis, MO), sonicated for 5 minutes to release spores from mother cells and incubated for 2 hours at 37ºC to digest the vegetative cells. To remove vegetative cell debris, spores were washed 10 to 14 times with 1/4 volume of deionized water followed by centrifugation at 2,050 g for 20 minutes. The purity of the spore suspension was verified with phase contrast microscopy to have less than 1% vegetative cell material.

Spore culturability

To determine the endospore culturability, heat-shock treatment at 80°C for 15 minutes was applied to a purified spore suspension with a known concentration that was first determined by microscopy as described above. A series of dilutions were made to reach an expected concentration range of 1000 spores/mL and 100 spores/mL. One hundred µL aliquots from each dilution were plated in triplicate on solidified growth medium (15 g agar per liter) and incubated in GasPak™ EZ Anaerobe pouch system (Becton Dickinson, Sparks, MD) at optimal growth temperature for 3 days. Colonies were counted and the average number was designated as colony forming units (CFU) per volume of original sample. The culturability was determined as the percentage of spores capable of forming colonies.

D-value measurement of spores

The D-value (i.e., decimal reduction time) was determined as the time required to kill 90% of spores at 100ºC. To determine the D-value of *C. sporogenes* a spore suspension was made to reach an expected concentration range of 100-1000 spores/mL where plating and incubation were done as detailed above. 2 mL of a dilute spore suspension was heated to 100 ºC, in a digital dry bath (Labnet International Inc., Edison, NJ). 100 µL samples were removed every 5 minutes and plated on solidified growth medium. Samples were also plated before heating to measure the original spore culturability. Samples were taken directly from the tube at 100°C to avoid temperature decrease during sampling. A similar procedure was followed for *C. hungatei*, however, samples were removed for plating every 1 minute.

Measurement of hydrophobicity

The hydrophobicity measurement is based on the partition of spores between an aqueous phase and a hydrocarbon phase. A spore suspension with an optical density (OD) between 0.4 and 0.5 as measured on a UV-Visible spectrophotometer (Varian Inc., Palo Alto, CA), was mixed with a 1/10 volume of hexadecane by vortexing for 30 seconds. The mixture was settled for 30 minutes to allow a hexadecane-aqueous partition to form. The upper phase was removed and the OD of the lower phase was recorded. The OD value of the hexadecane treated sample was divided by the original OD to get the hydrophobicity value of the spore suspension.

Release of DPA from spores of *C. sporogenes* and *C. hungatei* and quantification of DPA per spore using Tb^{3+}-DPA luminescence

A spore suspension in water of known concentration was autoclaved at 134 °C for 45 min at in a Tuttnauer 3870EA autoclave (Tuttnauer USA Co., Hauppauge, NY) to completely release DPA. After autoclaving, 10 μM $TbCl_3$ was added to spore suspension and DPA was detected using Tb^{3+}-DPA luminescence. Tb^{3+}-DPA luminescence excitation spectra (λ_{ex}=250-360 nm, λ_{em}=544 nm) and emission spectra (λ_{ex}=278 nm, λ_{em}=450-650 nm) were recorded with a fluorimeter model FL-1089 (Jobin Yvon, Edison, NJ) consisting of a 500-W Xe-lamp for excitation, two double-monochromators set at 4-nm bandpass, and a Pelletier-cooled photomultiplier tube model R928 (Products for Research, Inc., Danvers, MA). A 500 nm cut-off filter (Omega Filters, Brattleboro, VT) was placed at the entrance of the emission monochromator. Emission intensities recorded on different days were normalized to a Tb^{3+}-DPA standard solution. DPA released from spores was quantified by comparing the normalized intensity with a standard curve obtained by measuring the intensity of a known concentration range of pure DPA.

2.4 RESULTS

Production of *Clostridium* spores

For all three *Clostridium* species, sporulation commenced after 2 days of incubation, at which time premature spores appeared at the terminal ends of the vegetative cells. The cultures were incubated for a total of 5 to 6 days, which allowed spores to mature and the majority to be released from the mother cells. Our optimized sporulation procedure

allowed us to obtain pure spore suspensions of these three *Clostridium* species. Each batch of 75 mL of sporulated culture had a concentration of ~10^8 spores per mL (sp/mL). Upon subsequent purification and washing, 1 mL of a highly purified and concentrated spore suspension (10^{10} sp/mL) was obtained. Figure 2.1 shows the phase contrast microscopy image of purified spores of *C. sporogenes*, *C. hungatei* and the Greenland Ice Core isolate, *C. 5GA-1*. Spores can be seen as round phase bright bodies approximately 2.4 ± 0.3 μm in diameter for *C. sporogenes*. Spores of *C. hungatei* are elliptical, the size of which are 2.4 ± 0.3 μm wide and 3.6 ± 0.3 μm long. The average ratio of length to width is 1.55 ± 0.2. The spores of the Greenland isolate, *C.* G5A-1 are round shape, with approximately 2.4 ± 0.3 μm in diameter. The final purity of spores was greater than 99% with less than 1% vegetative cells observed as phase dark bodies.

Heat-shock treatment before incubation is extremely important for enhanced sporulation. Cultures of *C. sporogenes* without heat-shock resulted in only 45-50% sporulation, while those with heat-shock reached sporulation yields of 70-95% (Table 2.1). This effect of heat-shock treatment was even more striking with *C. hungatei*, but only when combined with a freeze-dried inoculum where heat-shock resulted in 75-90% sporulation, while non-heat-shocked cultures contained only 10% spores (Table 2.1). Freeze drying treatment is vital for *C. hungatei* sporulation. Our results showed that sporulation yields reached as high as 90% when a freeze-dried inoculum was used, while <1-5% sporulation was obtained without freeze drying (Table 2.1). The freeze drying treatment is also indispensable for endospore production from *Clostridium* G5A-1. Without freeze drying, the percentage of sporulation was less than 5% even after application of an appropriate

heat-shock treatment of 60 ºC for 15 min. However, when freeze drying was applied along with heat-shock treatment the percentage of sporulation reached 90% (Table 2.1). Incubation temperature affected the sporulation to a lesser extent than heat-shock and freeze drying treatments. The optimal growth temperatures for *C. sporogenes* and *C. hungatei* were 37 ºC and 30 ºC, while the suboptimal sporulation temperatures were 30ºC and 23 ºC, respectively. When sporulated at an optimal growth temperature, *C. sporogenes* reached 70% sporulation, while *C. hungatei* reached 75%. When the incubation temperature was decreased by 7 ºC to a suboptimal temperature, both species reached greater than 90% sporulation (Table 2.1). We also observed that when heat-shock treatment was combined with growth at suboptimal temperature (3 °C below the optimal growth temperature of 7 °C) and was applied to the psychrophilic *C. frigoris*, ~80% sporulation was achieved (data not shown). A high degree of sporulation makes the downstream harvest proceed with little difficulty. Heavy inoculum (~10%) is also necessary for high percentage sporulation since in most cases, an inoculum size less than 2% resulted in no growth in sporulation medium.

Characterization of spores of *C. sporogenes* and *C. hungatei*.

For a given concentration of *C. sporogenes* spores (measured by phase contrast microscopy), ~45% formed colonies on agar plates on the growth medium RCM, and only ~30% on tryptic soy agar (TSA). For a given suspension of *C. hungatei* spores, ~36% formed colonies on ATCC 2135 agar. The hydrophobicity of *C. sporogenes* and *C. hungatei* were 0.625 and 0.602 respectively. *C. sporogenes* is one of the most heat

resistant spore formers, with a D value of 15 min at 100 ºC; *C. hungatei* spores were much less heat-resistant with a D value of 5 min at 100 °C. Table 2.2 summarizes the properties of these two spore formers. DPA, a unique biomarker only found in endospores [27], was detected by Tb^{3+}-DPA luminescence where Figure 2.2 shows the spectral overlap of two different excitation spectra: an autoclaved spore suspension and a DPA reference control for both *C. sporogenes* and *C. hungatei*. This indicates that the spores of both *C. sporogenes* and *C. hungatei* contain DPA which can be released upon spore lysis by autoclaving. The lysis of spores was also confirmed by microscopy where spores turned from phase bright to phase dark under phase contract microscope after autoclaving. The average DPA content, determined by comparison with a DPA standard curve, was 3.7×10^8 and 3.6×10^8 molecules per spore for *C. sporogenes* and *C. hungatei,* respectively (Table 2.2). And the DPA content for *Clostridium* G5A-1 was 4.5×10^8 molecules per spore.

2.5 DISCUSSION

The diversity of the *Clostridium* genus provides a significant challenge to deriving a universal sporulation medium for these anaerobic endospore-forming microorganisms. This is exacerbated by characteristic unsynchronized sporulation and strain degeneration, which results in a low degree of sporulation. Moreover, the resistance of the tough cell wall to lysing agents makes the spore cleaning process very difficult. To overcome these problems, we have adopted a technique to synchronize the sporulation of two mesophilic *Clostridium* species resulting in a high concentration of clean *Clostridium* spores. The main factor for clean spore production is a high degree of sporulation, which can be

obtained by combinations of heat-shock, suboptimal sporulation temperature and freeze-dried inoculum.

We focused these initial efforts on optimizing the sporulation conditions of *C. sporogenes* and *C. hungatei*, two distinctive *Clostridium* species based on their G-C content (26% for *C. sporogenes* and 40% for *C. hungatei*), and detailed the sporulation outcomes for various combinations of heat-shock treatment, freeze-dried inoculum and suboptimal temperature incubation. When treatments that were optimal for sporulation of *C. sporogenes* and *C. hungatei* were applied to *C. frigoris* and *Clostridium* sp. G5A-1 we also observed high sporulation of 80% and 90% respectively, without further optimization. While the successful application of these conditions for these varied *Clostridium* species may indicate general utility, further species will need to be analyzed to verify this.

Various combinations of the three treatment options produced, in some cases, dramatically different sporulation outcomes. When the heat-shock treatment technique was applied alone the sporulation increased from 45% to 70% for *C. sporogenes*, but there was no increase for *C. hungatei*. Incubation at suboptimal temperature alone does not appear to significantly increase sporulation for either species; however, the combination of heat-shock treatment and growth at suboptimal temperature increased the percentage of *C. sporogenes* sporulation dramatically from 45% to 95% while only having a limited effect on *C. hungatei* with an increase from <1% to 5%. However, the

addition of a freeze-dried inoculum for *C. hungatei* increased sporulation to 90% when applied in combination with heat-shock and suboptimal temperature. The same effect was also observed with a Greenland Ice Core isolate, *Clostridium* G5A-1. When a freeze-dried inoculum of isolate G5A-1 was followed by heat-shock treatment, sporulation reached 90%, while heat-shock alone resulted in sporulation of <5%. We also found that the heat-shock temperature needed to activate synchronized sporulation varies with different species. For mesophilic species, 80 ºC was applied, while such a high temperature kills both vegetative cells and spores of the psychrophilic *C. frigoris*. In this case, a much lower temperature (40 ºC heat-shock treatment) was used to activate the culture. Our study indicates that combinations of these three treatments are necessary for high percentage sporulation and point to varied factors that control sporulation of *Clostridium*.

Strain degeneration (i.e., loss of the ability to sporulate) was observed in both *C. sporogenes* and *C. hungatei*. After 5 or 6 subcultures without heat-shock treatment, *C. sporogenes* no longer formed spores, while *C. hungatei* failed to sporulate after only 2 to 3 subcultures. We found that heat-shock treatment and suboptimal temperature incubation not only increased sporulation but also reduced the degeneration of sporulation. This is in agreement with Kashket and Cao, who concluded that heat-shock directly selects for non-degenerated cells while lowering the incubation temperature decreases the growth rate and thus the rate of acidification of the medium, allowing the sporulation pathway to be induced fast enough to outcompete degeneration. However, some degeneration resistant mutants have been isolated, which imply that the decrease in

degeneration may not simply be due to decreased growth rate and acidification, but a global gene regulatory mechanism may also play a role in strain degeneration [19]. We were able to recover the sporulation ability of degenerated *C. hungatei* and *Clostridium* sp. G5A-1 after the degenerated culture was freeze-dried and subsequently used as inoculum without heat-shock treatment to subsequently obtain a sporulation of 60-70% (data not shown). To date, there are no studies showing a correlation between freeze drying and sporulation.

A direct correlation of freeze drying to sporulation has not been previously documented. Freeze drying treatment of the inoculation, which contains a mixture of vegetative cells and spores, may affect sporulation by the up or down regulation of genes involved in the sporulation pathway in *Clostridium*. In *Bacillus subtilis*, the autostimulatory loop of spo0A results in a bistable gene expression which is responsible for the heterogeneity in the sporulating culture of this strain [28, 29]. If that is the case for *Clostridium* sporulation, freeze drying treatment may change the bistable distributions by up regulating the expression of spo0A, which results in high degree of sporulation. Desiccation has been shown to change the expression profiles of bacterial genes. For example, desiccation results in the up-regulation of genes responsible for drought survival for both *Bradyrhizobium japonicum* and *Rhodococcus jostii* [30, 31]. Freeze drying, heat-shock treatment and suboptimal growth conditions are all stressful situations for microorganism. The up-regulation of genes responsible for the survival of less than ideal condition is a natural response of microorganisms. Since sporulation is a mechanism of surviving conditions that are not suitable for growth it does not seem

surprising that the treatments used in this study would induce a subsequent sporulation response.

Quorum sensing, the ability of cells to sense their population density and communicate with each other and control cellular functions has been demonstrated in aerobic spore-forming *Bacillus* to regulate the sporulation pathway [32]. Whether or not quorum sensing plays a role in the sporulation of *Clostridium* species remains to be answered. Culture filtrates of a *Clostridium* strain, putrefactive anaerobe (PA) 3679 have been found to enhance sporulation of this species when adding to fresh media [33], and a sporulation factor has been found in the supernatant fluid of *C. perfringens* NCTC 8239 to stimulate sporulation of the same strain [34]. These results suggest *Clostridium* species may communicate with each other ("quorum sensing") during sporulation. L. Zhao *et al.* have shown that a molecule produced during *C. botulinum* growth has a similar chemical composition and structure with a quorum sensing signal molecule, AI-2 [35]. G. P. Carter *et al.* have identified a homologue of luxS (a gene that controls the synthesis of quorum sensing signal AI-2 in *Vibrio harveyi*) in the genome of *C. difficile* [36]. To date, quorum sensing has not yet been found to have any direct regulatory effect on the cellular function of *Clostridia*. It is also proposed that LuxS in *C. botulinum* may be simply a metabolic protein, while the signal molecule AI-2 is merely a metabolic product [36]. However, the inoculum size effect on sporulation, and the heat-shock synchronized growth shown in our results indicate quorum sensing may play a role in the sporulation of *Clostridium*. A large inoculum size could guarantee a high enough cell density for quorum sensing, while heat-shock treatment may activate some yet-to-be-found protein(s)

to synchronize the sporulating behavior of the cells in the culture. Our results contribute to an understanding of the varied and unknown factors that control sporulation of *Clostridia*.

In summary, for the case of *C. sporogenes*, inoculating with a large (10% v/v), post-log growth population, followed by heat-shock treatment and incubation under suboptimal temperature (30 ºC vs. 37 $ºC_{optimal}$) was sufficient to obtain a high percentage of sporulation. For the case of *C. hungatei*, the additional step of freeze drying the inocula was required to obtain high percentages of sporulation. To date, there are no previous studies showing a correlation between freeze drying and sporulation. Our results contribute to an understanding of the varied factors that control sporulation of *Clostridium*.

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Table 2.1. Effect of different combinations of three sporulation conditions on sporulation of difference *Clostridium* species.

Sporulation Conditions			Species		
Freeze Dry	Heat-shock	Suboptimal T	*C. sporogenes*	*C. hungatei*	*C.* G5A-1
–	–	–	45%	<1%	<1%
–	–	+	50%	<1%	nd
–	+	–	70%	<1%	<5%
–	+	+	95%	5%	nd
+	–	+	nd	10%	nd
+	+	–	nd	75%	90%
+	+	+	nd	90%	nd

nd: not determined

Table 2.2. Properties of *Clostridium* spores.

	C. sporogenes		*C. hungatei*
Optimal growth temperature	37ºC		30ºC
Optimal Sporulation temperature	30ºC		23ºC
Spore size (μm)	2.4 diameter		3.6 × 2.4
DPA content (molecules/spore)	3.7×10^8		3.6×10^8
Culturability	45% (RCM)[a]	30% (TSA)[b]	36%[c]
$D_{100ºC}$ Value	15 min		5 min
Hydrophobicity	0.625		0.602

a. *C. sporogenes* spores wereincubated on RCM (reinforced clostridial medium) agar.

b. *C. sporogenes* spores were incubated on TSA (tryptic soy agar).

c. *C. hungatei* spores were inculbated on ATCC 2135 agar.

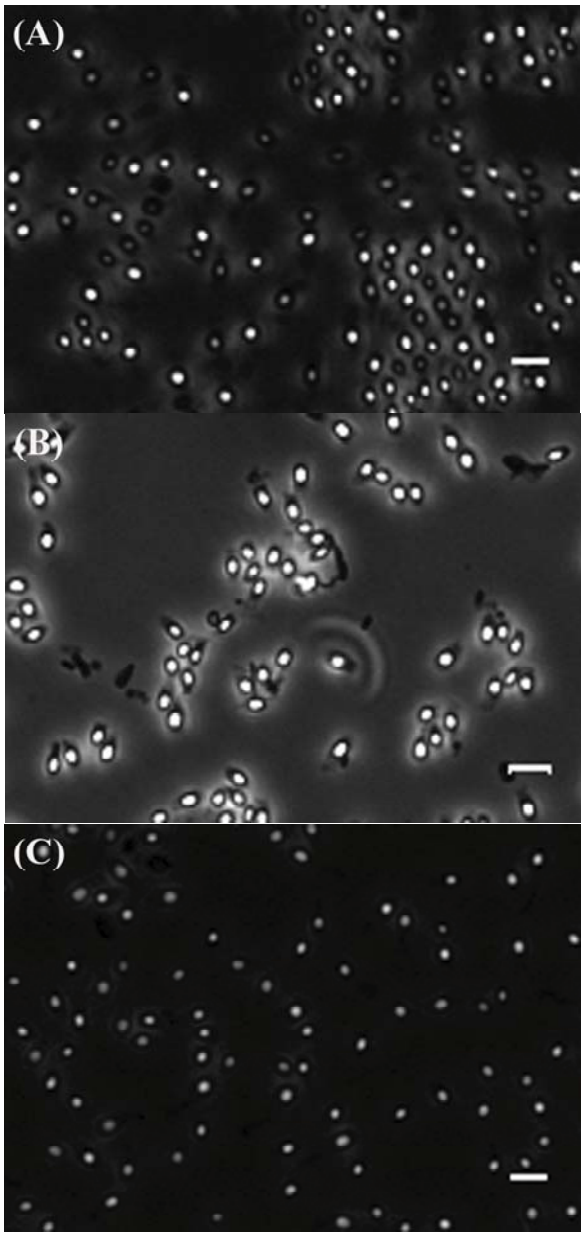

Figure 2.1 Phase contrast microscope image of (A) *C. sporogenes*, (B) *C. hungatei* and (C) *Clostridium* G5A-1 spores. Scale bar: 10 μm.

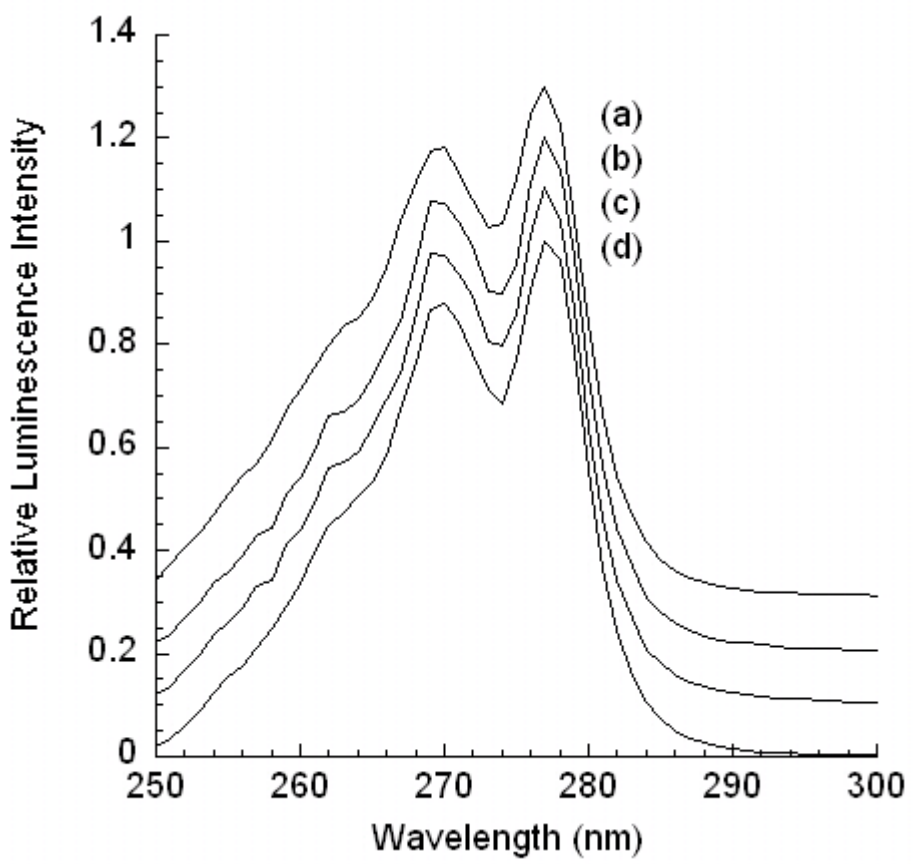

Figure 2.2 Excitation spectra (λobs=543 nm) autoclaved spores (10^6 spoes/mL) from (a) *C. sporogenes*, (b) *C. hungatei* and (c) *Clostridium* G5A-1, and (d) DPA reference (1 μM) with $[Tb^{3+}]$ = 10 μM. Spectra are offset for clarity.

CHAPTER 3: RAPID ENDOSPORE VIABILITY ASSAY FOR *CLOSTRIDIUM* SPORES[†]

3.1 ABSTRACT

A rapid spectroscopy based Endospore Viability Assay (Spectro-EVA), previously developed for *Bacillus* spores, was modified for enumeration of germinable *Clostridium sporogenes* spore. The Spectro-EVA is based on the detection of dipicolinic acid (DPA), which is released during stage I germination and quantified by terbium ion (Tb^{3+})-DPA luminescence. Germination of *C. sporogenes* spores in aqueous suspension was induced by L-alanine and $NaHCO_3$ addition, and germinable endospores concentrations were determined by reference to a standard curve. Determination of the fractions of germinable *C. sporogenes* spores by Spectro-EVA and phase-contrast microscopy yielded comparable results of 54.0% ± 2.9% and 59.3% ± 2.6%, respectively, while only 32.3 % ± 5.3% of spores produced colonies on reinforced clostridial medium (RCM). Rates of germination were measured as a function of temperature (30 °C~60 °C) using Spectro-EVA, yielding a linear relationship between the square root of the rate constant and inverse temperature. Spectro-EVA was also applied to study the effect of D-alanine, a germination inhibitor for most *Bacillus* spores, on the germination of *Clostridium* spores. Our results showed that while D-alanine inhibited the germination of various *Bacillus* spores, it germinated *Clostridium* spores at a similar germination rate and extent

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W.-W. Yang[1] and A. Ponce[1,2]
[1]California Institute of Technology, Pasadena, CA 91125
[2]Jet Propulsion Laboratory, Pasadena, CA 91101

as L-alanine. These findings indicate that Spectro-EVA could potentially be used to distinguish aerobic and anaerobic spores.

3.2 INTRODUCTION

Bacterial spores (endospores) are dormant cellular structures in the life cycle of *Bacillus* [1, 2] and *Clostridium* [2, 3] and are arguably the hardiest life forms on Earth [4-8]. Given the importance of bacterial spores in various industries as illustrated in chapter 1, a rapid and accurate method to detect and quantify viable spores in any given samples is desirable.

Currently, the standard method to quantify endospore viability is based on culture dependent methods such as determination of numbers of colony forming units (CFU) from plate counts, which requires several days of incubation and tedious anaerobic techniques for *Clostridium* species. To facilitate quantification, Shafaat and Ponce (2006) developed a spectroscopy based Endospore Viability Assay (Spectro-EVA) for *Bacillus* spores based on the detection of a unique biomarker, dipicolinic acid (DPA). Despite exhibiting no detectable metabolic activity, spores are able to monitor the nutritional status of their environment and germinate when conditions are favorable [9-12]. When specific germinants are added to a spore suspension, spores proceed towards germination and outgrowth *via* the following steps: (1) spore activation, (2) stage 1 germination, during which water partially rehydrates the spore core and DPA is released, (3) stage 2 germination, during which cortex hydrolysis and metabolic activity resumes, and (4)

outgrowth [9, 11, 13]. DPA, released from the spore core during stage 1 germination, can be detected by adding $TbCl_3$ to the spore suspension and measuring the resulting Tb^{3+}-DPA luminescence intensities (Figure 3.1) [14-17]. Spore can also be lysed and DPA is released from spore core by physical forces, such as microwaving and autoclaving.

The DPA concentration can be correlated to the germinable spore population, given an average DPA content per spore, and full DPA release during germination [10, 13, 18]. This is the underlying principle of Spectro-EVA, in which the germinable spore population is determined by measuring the proportion of DPA released after germination versus DPA released from the total spore population after lysis by autoclaving.

We have adapted Spectro-EVA, previously used for *Bacillus* spores, to quantify total numbers of *Clostridium* spore and the fractions of these spores that could germinate. Spectro-EVA has been validated against traditional methods of enumeration by phase contrast microscopy and plate counts and applied to study the germination dynamics of *C. sporogenes* spores as a function of temperature. Spectro-EVA has also been applied to study the effect of D-alanine, a germination inhibitor of *Bacillus* spores, on the germination of *Clostridium* spores.

3.3 MATERIALS AND METHODS

Materials

Deionized water (18.2 MΩ/cm) was obtained from an ultrafilter system (Water Pro PS, LabConco, Kansas City, MO, USA). Terbium (III) chloride hexahydrate-(99.999%), dipicolinic acid (2,6-pyridinedicarboxylic acid, DPA, 99%), L-alanine and L-lactate were purchased from Aldrich (Milwaukee, WI, USA). *C. sporogenes*, ATCC 7955 (American Type Culture Collection, Manassas, VA, USA) was obtained as a freeze-dried pellet. *B. atrophaeus* (ATCC 9372) endospores were purchased from Raven Biological laboratories. Endospore suspension was stored at 4 ºC in the dark before use.

Endospore production and purification

C. sporogenes was revived in approximately 5 mL of reinforced clostridial medium (RCM; Becton Dickinson, Sparks, MD, USA) at 37 ºC for 3 days. The culture was transferred to fresh medium after development of visible turbidity. The production and purification of *C. sporogenes* spores was performed using a protocol developed specifically for *Clostridium* species [19]. The spore suspension contained less than 1% vegetative cell material as determined by phase contrast microscopy.

Endospore quantification by Tb^{3+}-DPA luminescence

Tb^{3+}-DPA luminescence excitation spectra (λ_{ex}=250-360 nm, λ_{em}=544 nm) and emission spectra (λ_{ex}= 278nm, λ_{em}= 450-650 nm) were recorded with a fluorimeter (Model FL-1089, Jobin Yvon, Edison, NJ, USA) consisting of a 500-W Xe-lamp for excitation, two

double-monochromators set at 4-nm bandpass, and a Pelletier-cooled photomultiplier tube (Model R928, Products for Research Inc, Danvers, MA, USA). A 500 nm cut-off filter (Omega Filters, Brattleboro, VT, USA) was placed at the entrance of the emission monochromator. Emission intensities recorded on different days were normalized by reference to the emission intensity of a Tb^{3+}-DPA standard solution.

To correlate emitted intensity of luminescence with spore concentration, 13 triplicate suspensions of spores at numbers ranging from 10^6 to 10^9 spores/mL were autoclaved at 136 °C for 60 minutes to completely release DPA from spores. The luminescence intensity obtained with each spore suspension was then recorded and a calibration curve was obtained (Figure 3.2). The possibility of interference of germinants on the luminescence intensity was eliminated by adding to suspensions the same concentrations of L-alanine (100 mM) and $NaHCO_3$ (50 mM) as were used in validation experiments described below.

Spectro-EVA determination of germinable and total spore concentrations

To determine the number of germinable spores in samples, germination was induced in triplicate samples of suspensions containing known total numbers of spores by adding 0.3 mL of 1 M L-alanine and 0.15 mL of 1 M $NaHCO_3$ to 2.55 mL of each spore suspension. The final concentrations of germinants were 100 mM for L-alanine and 50 mM for $NaHCO_3$. The supplemented suspensions were then incubated at 37 °C for 48 h to allow the germination process to go to completion. Following germination, 30 µL of 1 mM $TbCl_3$ was added to each suspension to obtain a final concentration of 10 µM $TbCl_3$.

Tb^{3+}-DPA luminescence emission and excitation spectra of the supsensions were recorded. The luminescence intensity was converted to the number of germinable spores/mL by reference to the calibration curve (Figure 3.2).

To determine the total number of spores, each supsension use for determination of germinable spore numbers was sealed in a microwavable tube and autoclaved at 136 °C for 60 minutes, to release DPA from spores that had not germinated. After cooling to room temperature, the supsensions were transferred to plastic cuvettes, and Tb^{3+}-DPA luminescence emission and excitation spectra were determined. The total numbers of spores/mL were obtained by reference to the calibration curve. The proportion of spores that germinated was then calculated as the ratio of germinated spores to total spores.

Phase contrast microscopic enumeration of total and germinable spores

Five μL aliquots of spore suspensions were placed in a Petroff-Hausser counting chamber (Model 3900, Hausser Scientific, Horsham, PA, USA), and spores were observed at 400× magnification using a phase contrast microscope (Nikon Eclipse 80i, AG Heinze Co, Lake Forest, CA, USA) fitted with a digital camera (Nikon Digital Sight DS-5M). The smallest squares in the counting chamber are 0.05 mm × 0.05 mm, of which 80 were analyzed to obtain the average spore count per square. To obtain statistically significant counts, the spore concentration was kept above 10^7 spores/mL, which resulted in at least 10 cells per sixteen squares. Spore suspensions with numbers in the range 5×10^7- 5×10^8 spores/mL were prepared by appropriate dilution of the stock suspension. Non-

germinated, intact spores are phase bright, while germinated spores and vegetative cells are phase dark under phase contrast illumination. Phase bright spores were counted before and after germination. The total numbers of spores were determined from counts of phase bright spores before germination, while the numbers of germinable spores were determined from the differences in the numbers of phase bright spores before and after germination.

Determination of CFU per milliliter of spore suspension

A heat-shock treatment of 80 °C for 15 minutes was applied to a spore suspension for which the total number of spores had been determined by phase contrast microscopy. Dilutions were prepared to obtain spore suspensions containing between 10^2 and 10^3 spores/mL. One hundred µL aliquots from each dilution were plated in triplicate on RCM supplemented with agar at 15 g/L. The plates were incubated in a GasPak™ EZ Anaerobe pouch system (Becton Dickinson) at 37 °C for 3 days. Colonies were counted and the mean number of CFU/mL in each original sample was calculated.

Germination dynamics of temperature dependence

The temperature dependence of spore germination over time was determined by measuring the Tb^{3+}-DPA luminescence intensity increase as spores germinated (Figure 3.3). Spore suspensions containing ~10^7 spores/mL, supplemented with 100 mM L-alanine, 10 mM $NaHCO_3$ and 10 µM $TbCl_3$, were incubated at 30 °C, 37 °C, 45 °C and 60 °C. The germinants, L-alanine and $NaHCO_3$, were added to the spore suspensions at time zero. Suspensions were buffered by 500 mM sodium acetate buffer at pH 6.5. Tb^{3+}-

DPA luminescence emission spectra intensity of each supsension was recorded at various intervals during incubation until the luminescence intensity reached a plateau. The plateau is reached when all germinable spores have germinated and completely released DPA under the germination conditions provided. Spore germination time courses were modeled using $G_t = A - e^{-rt}$ [28], where G_t is the luminescence intensity at time t, A is the plateau luminescence intensity, and r is the germination rate constant. The best fit to the experimental data yielded the plateau intensity parameter (A) for each incubation temperature. The time course data in terms of extent of germination was obtained by normalizing the luminescence intensity time course data with respect to A.

Comparison of the effect of D-alanine on *Clostridium* and *Bacillus* spore germination

Competition experiments were conducted to spores of three *Clostridium* species (*C. sporogenes, C. hungatei* and a Greenland Ice Core isolate, *C. G5A-1*) and three *Bacillus* species (*B. atrophaeus, B. subtilis* isolated from Atacama Desert, and *B. simplex* isolated from Kilimanjaro) with different concentration combinations of L-alanine and D-alanine. Spore suspensions containing ~5×10^6 spores/mL, supplemented with 100 mM germinants (L-alanine and D-alanine at various ratios, while the total germinant concentrations were constant) and 10 μM $TbCl_3$, were incubated at 37 °C. The ratios of D-alanine to L-alanine used were as follow: 9:1, 3:1, 1:1, 1:3, and 1:9. Spore suspensions supplemented with only D-alanine (100 mM) or L-alanine (100 mM) were also used as controls. The germinants were added to the spore suspensions at time zero. Suspensions were buffered by 500 mM sodium acetate buffer at pH 6.5. Tb^{3+}-DPA luminescence emission spectra

intensity of each suspension was recorded at various intervals during incubation until the luminescence intensity reached a plateau. Following germination, each suspension was sealed in a microwavable tube and autoclaved at 134 °C for 60 minutes. The Tb^{3+}-DPA luminescence intensity at each interval was normalized to the intensity measured after autoclaving with the same suspension.

3.4 RESULTS

Correlation of Tb^{3+}-DPA luminescence intensity with spore concentration

The plot of Tb^{3+}-DPA luminescence intensity versus microscopic enumeration of spores exhibited a linear correlation (R^2 = 0.999) for spore numbers in the range 10^6 to 10^9 spores/mL (Figure 3.2). On the assumption that there is an average DPA content per spore, the average DPA content per *C. sporogenes* spore was calculated to be 3.7×10^8 molecules per spore, which is comparable to the value of $1.7\sim2.2 \times 10^8$ molecules per spore for *Bacillus* spores [14, 17].

The release of DPA from spores upon germination

We have tested the effects of a variety of germinants and germinants combinations on *C. sporogenes* germination. These germinants include L-alanine, L-lacte and $NaHCO_3$. For optimal germination of *C. sporogenes* spores, a combination of 100 mM L-alanine and 50 mM $NaHCO_3$ was required. *Clostridium* spores also germinated upon the addition of L-lactate, but at a much slower rate and to a lesser extent (data not shown). Even under

optimized germination conditions, germination of the tested strain of *C. sporogenes* is approximately 10 times slower than that of *Bacillus atrophaeus* [17].

On addition of L-alanine and $NaHCO_3$ to a *C. sporogenes* spore suspension, DPA was released from the core of the germinated spores into the bulk solution (Figure 3.3). Tb^{3+}-DPA luminescence intensity increased over time and reached a plateau at approximately 60% after 30 h. The phase contrast image after germination also shows approximately 60% spore germination. Figure 3.4 shows the transition in one sample from phase bright to phase dark before and after germination, respectively.

Validation of spectroscopy based endospore viability assay (Spectro-EVA)

To validate Spectro-EVA, we applied Spectro-EVA, phase contrast enumeration and CFU counts on the same spore suspension to measure germinability/viability, the percentage of spores that germinated after two days of incubation. The percentage of spores that germinated after two days of incubation was 54.0% ± 2.9% determined by phase contrast microscope enumeration and 59.3% ± 2.6% by Spectro-EVA. For the same spore suspension, only 32.3% ± 5.3% formed colonies on RCM agar plates incubated anaerobically at 37 °C after three days (Figure 3.5).

To further validate Spectro-EVA, a set of side-by-side comparison experiments between Spectro-EVA and phase contrast enumeration was performed over the concentration range of 10^6-10^7 spores/mL. There was a one-to-one correlation between phase-contrast

and Spectro-EVA determination of the total and germinable spores/mL, indicated by a good fit to a line with a slope of unity (Figure 3.6). This validated the Spectro-EVA methodology with respect to the traditional phase contrast microscopy methodology to enumerate total and germinable spores over a range of 10^6-10^7 spores/mL in spore concentration. These data show that the Spectro-EVA we developed for *Clostridium* spores can be used to quantify the concentration of total and germinable spores in a given sample.

Temperature dependence of germination rates

The germination dynamics were accurately described by the equation for G_t for each temperature shown (Figure 3.7A, $R^2 > 0.99$ for each time course). From the best fit, we obtained the germination rate (r), which increased as temperature (T) increased from 30 ºC to 60 ºC. The temperature dependence data yielded a linear relationship between √r and 1/T (Figure 3.7B). This shows that we can estimate the germination rate at any temperature between 30 ºC and 60 ºC. This model predicts that germination would cease at 12 ºC, however, at 4 ºC we still observed minimal germination and release of DPA (data not shown). This may be due to different germination dynamics at lower temperatures.

Effect of D-alanine on spore germination of *C. sporogenes* and *B. atrophaeus*

Figure 3.8 showed the effect of D-alanine on the germination of various *Bacillus* and *Clostridium* spores. For a given *Clostridium* spore suspension, when the concentration

ratio of D-alanine to L-alanine increased while the total germinant concentration remained constant (100 mM), the germination rates and extent remained constant (Figure 3.8 D, E, F). However, when the same experiment conducted with *Bacillus* spores, we observed a different pattern. *B. subtilis* only germinated in 100 mM L-alanine, while replacing 10% of L-alanine with D-alanine completely inhibited spore germination (Figure 3.8 A). As for *B. atrophaeus*, D-alanine started to inhibit spore germination when the concentration ratio of D-alanine to L-alanine exceeded one, indicated by the slower germination rate and lesser germination extent. When D-alanine served as the sole germinant, we still observed the release of DPA from *B. atrophaeus* spores; however, it may be due to the spontaneous release of DPA, not through spore germination, since DPA was released from *B. atrophaeus* spores without the presence of any germinant at a faster rate and a higher extent (Figure 3.8 B). Our results also showed that D-alanine does not have obvious inhibitory effect on the germination of *B. simplex* spores (Figure 3.8 C). This indicated that D-alanine could be used interchangeable as a germinant with L-alanine for *Clostridium* spores, but not for *Bacillus* spores.

3.5 DISCUSSION

We have developed a spectroscopy based Endospore Viability Assay (Spectro-EVA) to detect anaerobic spores, and to measure the germinable spores and total spores in aqueous suspensions. Spectro-EVA has also been validated with respect to traditional phase contrast microscopy method and CFU measurement, which shows comparable results with these three methods (Figure 3.5). Spectro-EVA measurements probe DPA release during stage 1 germination, while phase contrast microcopy measurements probe water

uptake by spores which leads to the loss of refractivity under phase contrast microscope. CFU measurements probe the cell division, which is the last stage of the germination process, and requires proper growth conditions (e.g., enough nutrient, and anaerobic environment for *Clostridium* spores). The advantages of Spectro-EVA over phase contrast microscopy and CFU measurements are that it only requires simple analytical method to quantify DPA release by Tb^{3+}-DPA luminescence, and it probes for viability at an earlier stage so as to avoid the specific growth requirements and longer time for full outgrowth.

Endospore germination was assumed to indicate spore viability. In any spore population, a fraction of the total spores is germinable, and a fraction of germinable spores will give vegetative cells that will grow to form colonies. Within the germinable population, a subset may be germinable-but-not-viable, and within the viable spore population, subsets may be viable-but-not-germinable (i.e., superdormant), viable-but-not-culturable (VBNC), and viable-and-germinable-but-not-culturable. CFU measurement will most likely underestimate the number of viable spores due to the existence of VBNC spores [20]. In contrast, EVA will most likely overestimate the number of viable spores due to the existence of germinable-but-not-viable spores, although the possibility of superdormant endospore populations would reduce that overestimation.

From the germination kinetics study, we observed a linear correlation between $\sqrt{r}$ and 1/T. This leads to the definition of a theoretical lowest temperature that supports

germination and outgrowth of *Clostridium* spores, at which germination rate r equals to zero. The theoretical lowest temperature could be used as the upper limit of the food processing temperature to avoid the potential survival and growth of *C. botulinum* spores. However, germination was observed at a temperature below the theoretical lowest point, which suggests the germination dynamics may be different from the model at lower temperatures. In this case, the relation of $\sqrt{r}$ and 1/T may not be linear any more at lower temperatures. If this is true, in the food processing, the upper limit of processing temperature should be set to be a couple degree lower than the theoretical temperature.

D-alanine has been reported as a germination inhibitor for *Bacillus* spores by competing against with L-alanine for the binding site on the spore coat [21-23]. However, these studies have not been conducted with *Clostridium* spores. We have shown that D-alanine can be interchangeable with L-alanine as a germinant for *Clostridium* spores, while it inhibits the germination of 2 out of 3 *Bacillus* spores tested. D-alanine can be potentially served to distinguish *Clostridium* spores from *Bacillus* ones. Our results also indicate that the difference between *Bacillus* and *Clostridium* spores may lie in the different properties of the binding site on the spore coat of these two genera.

The possible survival and outgrowth of various *Clostridium* spores during processing and storage of food is of concern. Because *Clostridium* spores are more resilient than other pathogens, they are employed as biological indicators for evaluating the efficacy of sterilization regimens [25, 26]. Ultimately, we envision that a further optimized Spectro-EVA protocol for *Clostridium* spores will be applied to evaluate the efficiency of

different food processing procedures. Moreover, *C. sporogenes* is also a great surrogate for *C. botulinum* because it is a common spoilage organism found in sealed products that are maintained under anaerobic conditions. As new processing technologies, such as ultra-high pressure processing (UPP), ionizing radiation, high voltage electric discharges, microwave process, are being considered, we envision that Spectro-EVA will be employed to monitor the inactivation of a surrogate *C. sporogenes* spores to the process. Spectro-EVA also has the potential to monitor the in situ inactivation dynamics, which would be helpful in validating a given processing technology. Given these factors, we believe that Spectro-EVA is poised to provide a faster and more accurate way to design inactivation regimens and monitor the efficiencies of various new and emerging food preservation technologies as they continue to expand [27].

Other potential applications of Spectro-EVA for *Clostridium* endospores include evaluating the efficacy of sterilization regimens for wastewater and medical equipment. In the waste water treatment industry, spores of sulfite-reducing clostridia (SSRC) have been considered as an indicator for the presence of pathogenic microorganisms. We envision that Spectro-EVA will be applied to measure the viable SSRC before and after treatment to evaluate the efficiency of different treatments. In the medical field, there is also strong evidence suggesting that contact transmission from contaminated articles or the hands of staff is responsible for the spread of diseases. This is due to the persistence of spores of *C. difficile* on inanimate articles, such as lamps, door handles or bed rails. Measurements that guarantee rooms are thoroughly cleaned between patients are indispensable in preventing the transmission of this disease. We envision that Spectro-

EVA will be applied to evaluate the effectiveness of any process that is used to sterilize surfaces in the hospital.

In summary, we have developed a Spectro-EVA to detect and quantify *Clostridium* spores. Spectro-EVA has been validated against phase contrast microscopy and CFU measurements, which indicates that Spectro-EVA can be used as a rapid and simpler alternative to CFU measurement for quantifying *Clostridium* spores. Spectro-EVA has been applied study the germination kinetics *C. sporogenes* spores, and a theoretical lowest germination temperature was derived. EVA has potential applications in food industry, waste water treatment and astrobiology due to its significant importance in these areas.

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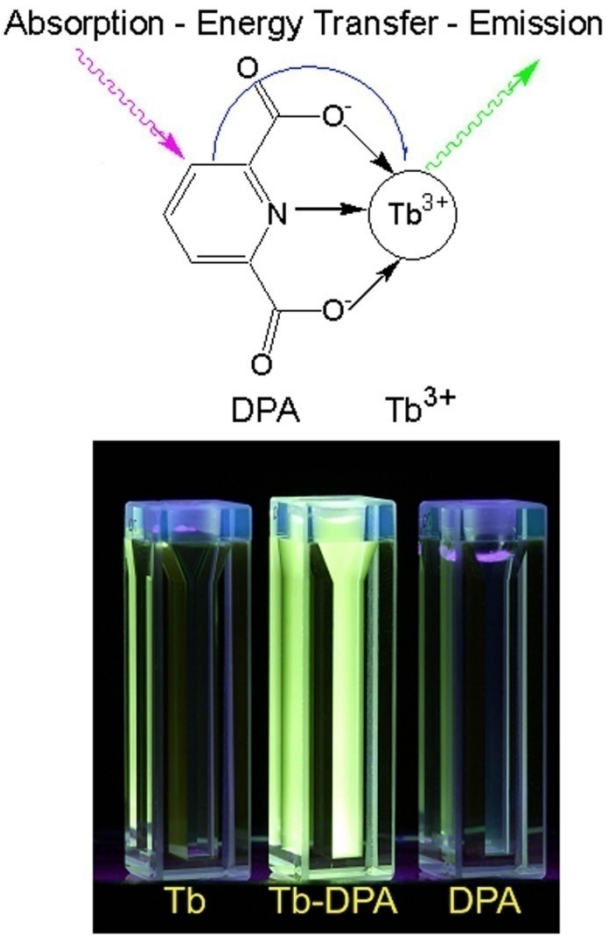

Figure 3.1 (A) Absorption-energy transfer-emission mechanism for the Tb^{3+}-DPA luminescence assay. (B) Cuvettes containing solutions of 1 mM Tb^{3+}, 1 mM Tb^{3+} plus 1 mM DPA, and 1 mM DPA under UV (254 nm) light.

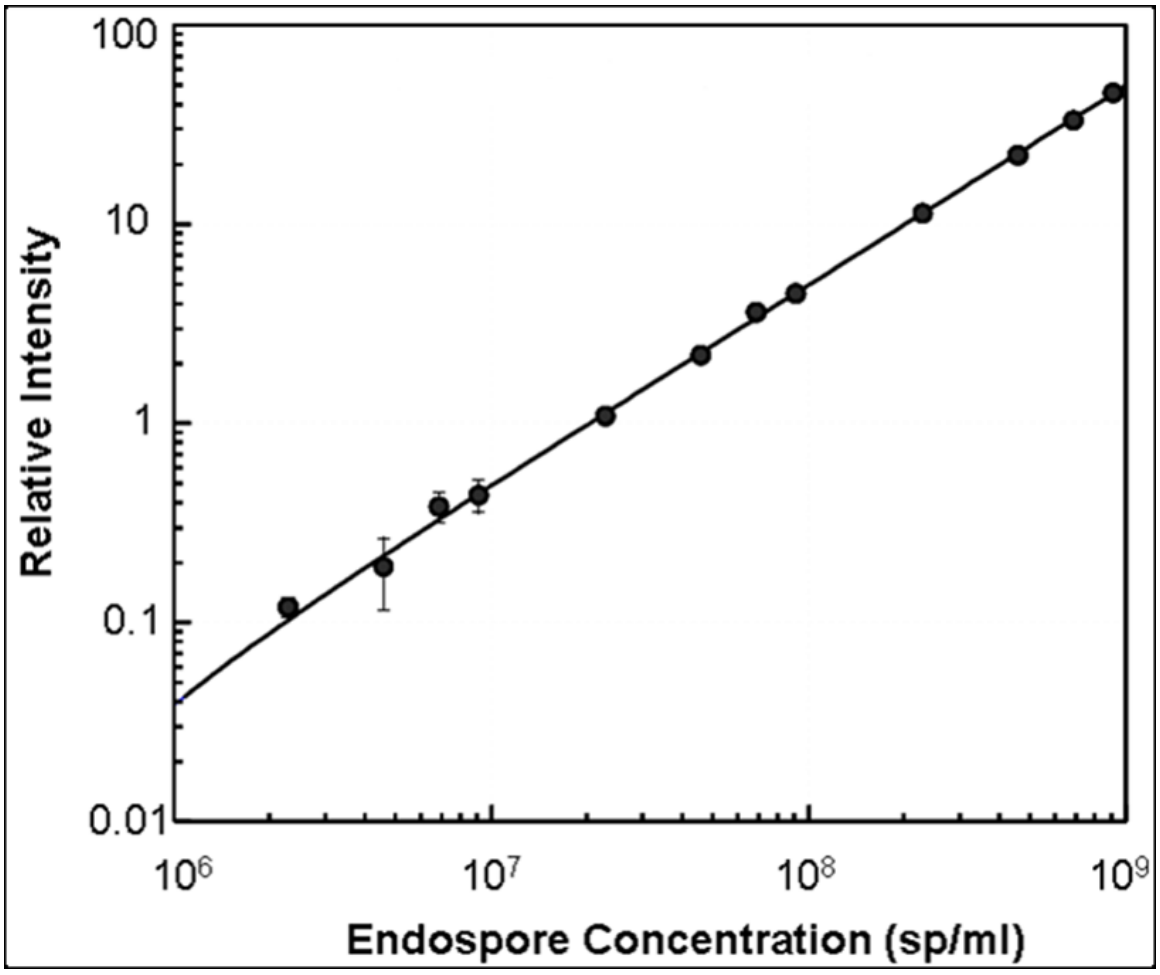

Figure 3.2 Tb^{3+}-DPA luminescence intensity standard curve. Error bars represent standard deviation.

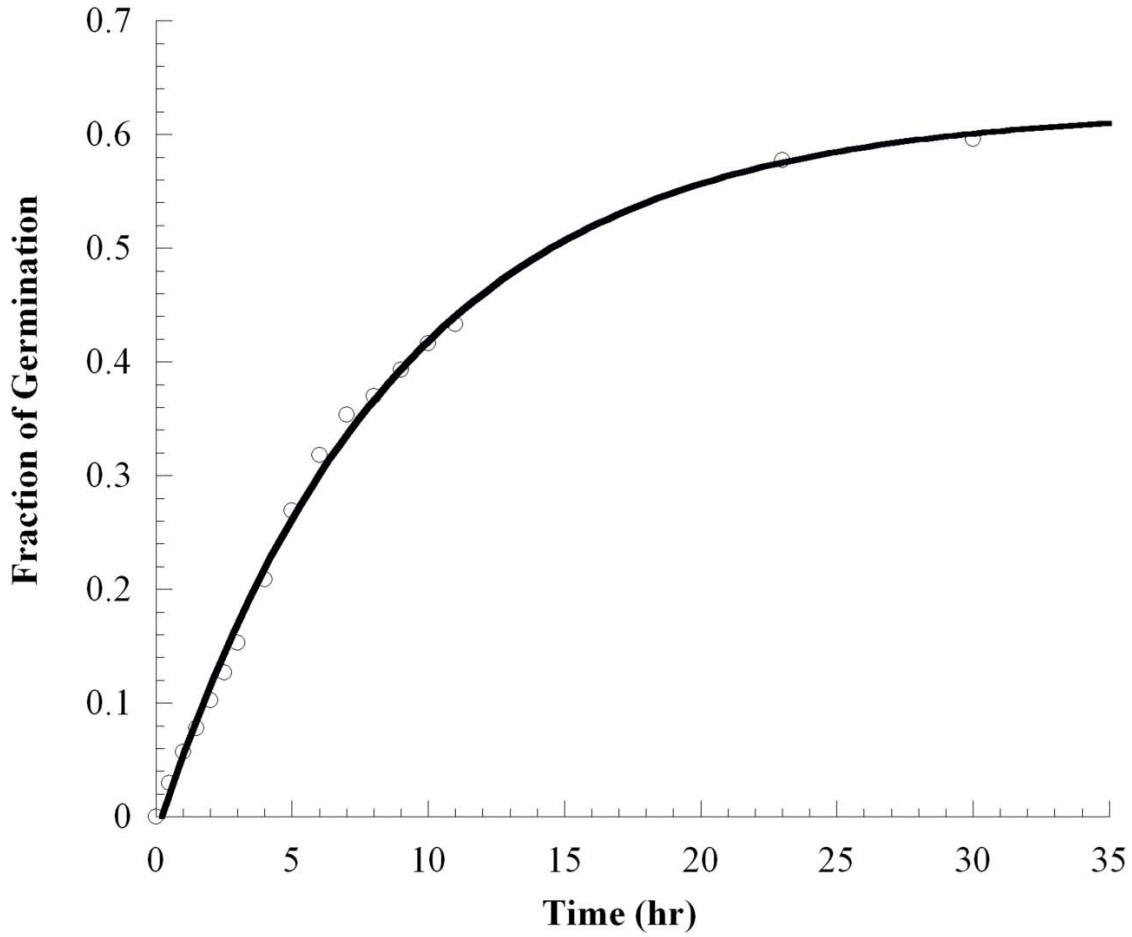

Figure 3.3 Germination timecourse of *C. sporogenes* spores, which shows Tb^{3+}-DPA luminescence intensity as a function of time.

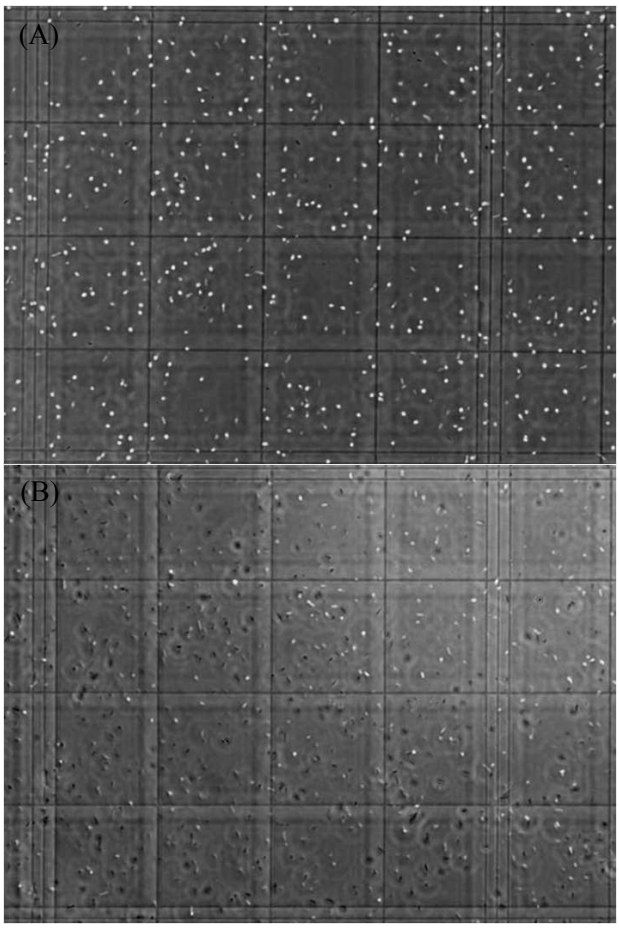

Figure 3.4 Phase contrast images of spores, which appear as (A) phase bright bodies when intact, and (B) after germination is complete.

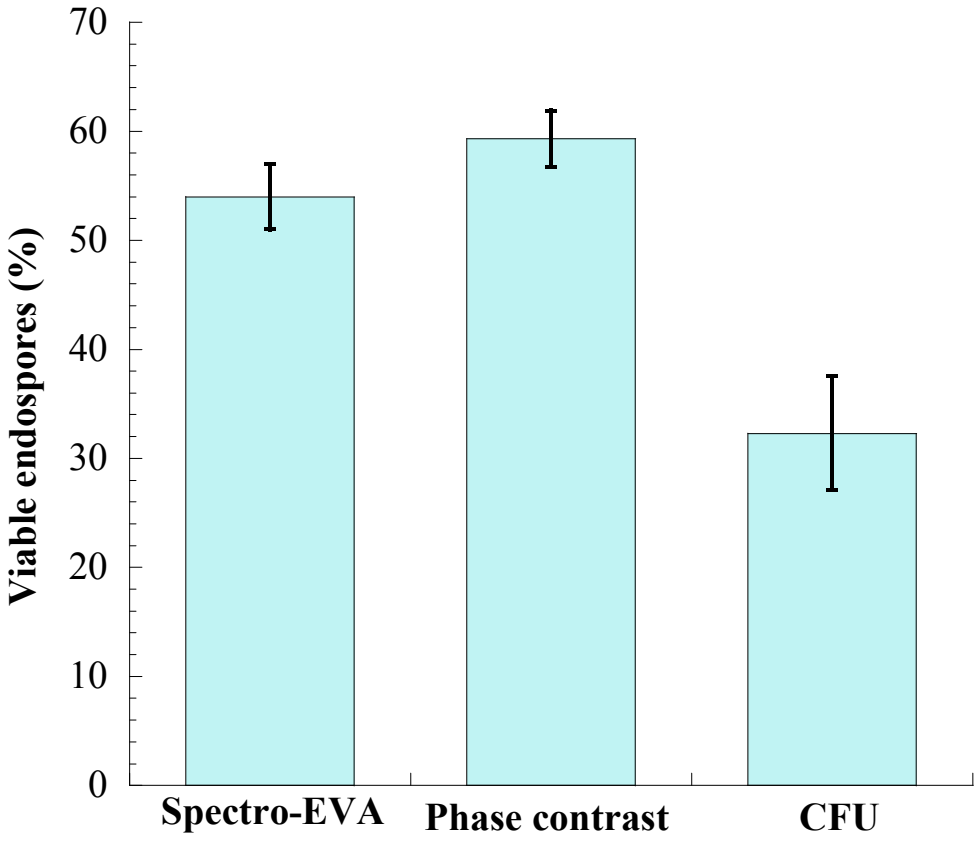

Figure 3.5 Percentage of germinable spores in the total population that can be determined by Spectro-EVA, phase contrast microscopy, and CFU enumeration.

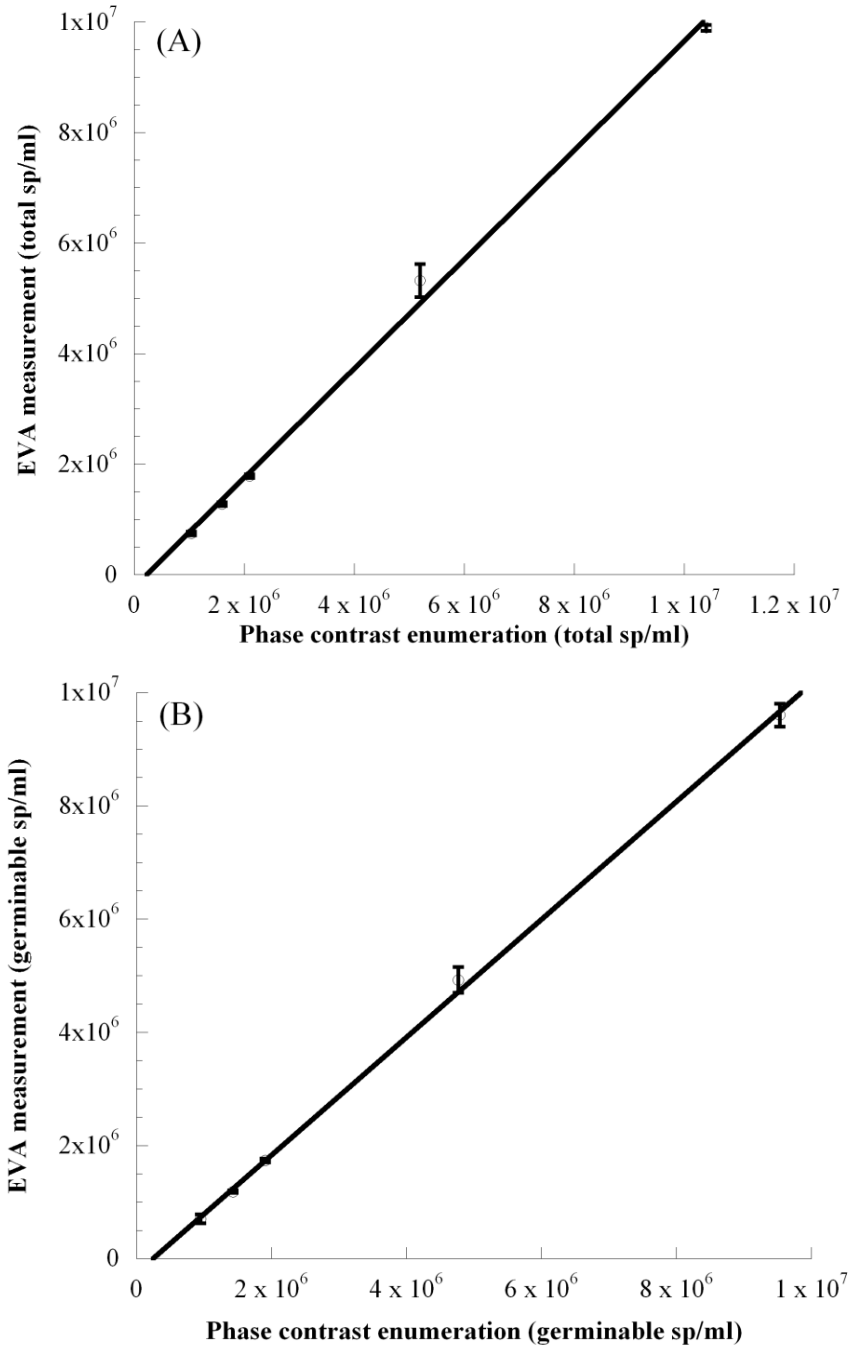

Figure 3.6 (A) Correlation between phase-contrast enumeration and Tb^{3+}-DPA luminescence assay for quantification of total spore concentration. (B) Correlation between phase contrast enumeration and Tb^{3+}-DPA luminescence assay for quantification of germinable spores. The lines shown indicate a 1:1 correspondence.

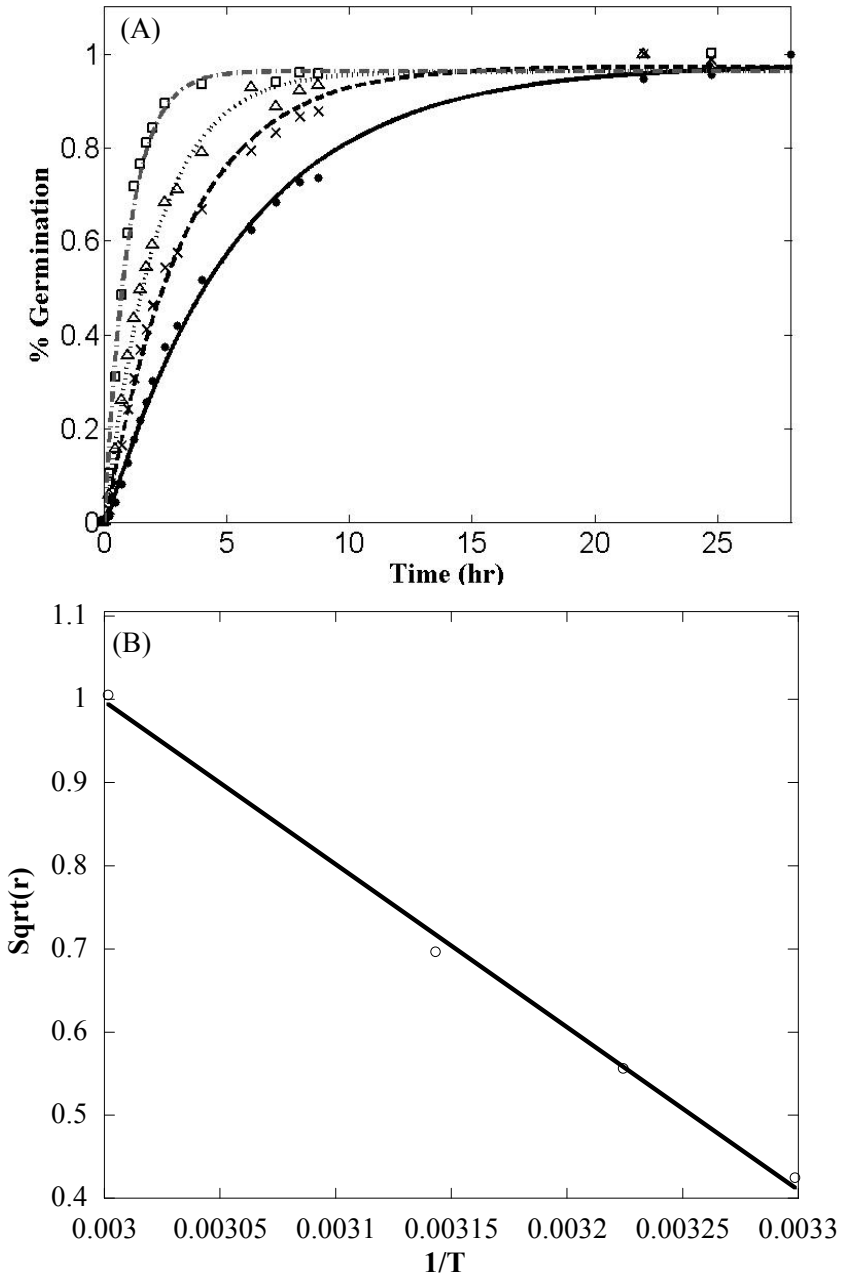

Figure 3.7 (A) The extent of germination over time at temperatures: 30 °C (solid circles), 37 °C (crosses), 45 °C (triangles) and 60 °C (squares). Points on graph are experimental data, and lines indicate fit to germination model, $G_t = A - e^{-rt}$. (B) Linear relation between 1/T and √r, where T is the germination temperature and r is the corresponding germination rate.

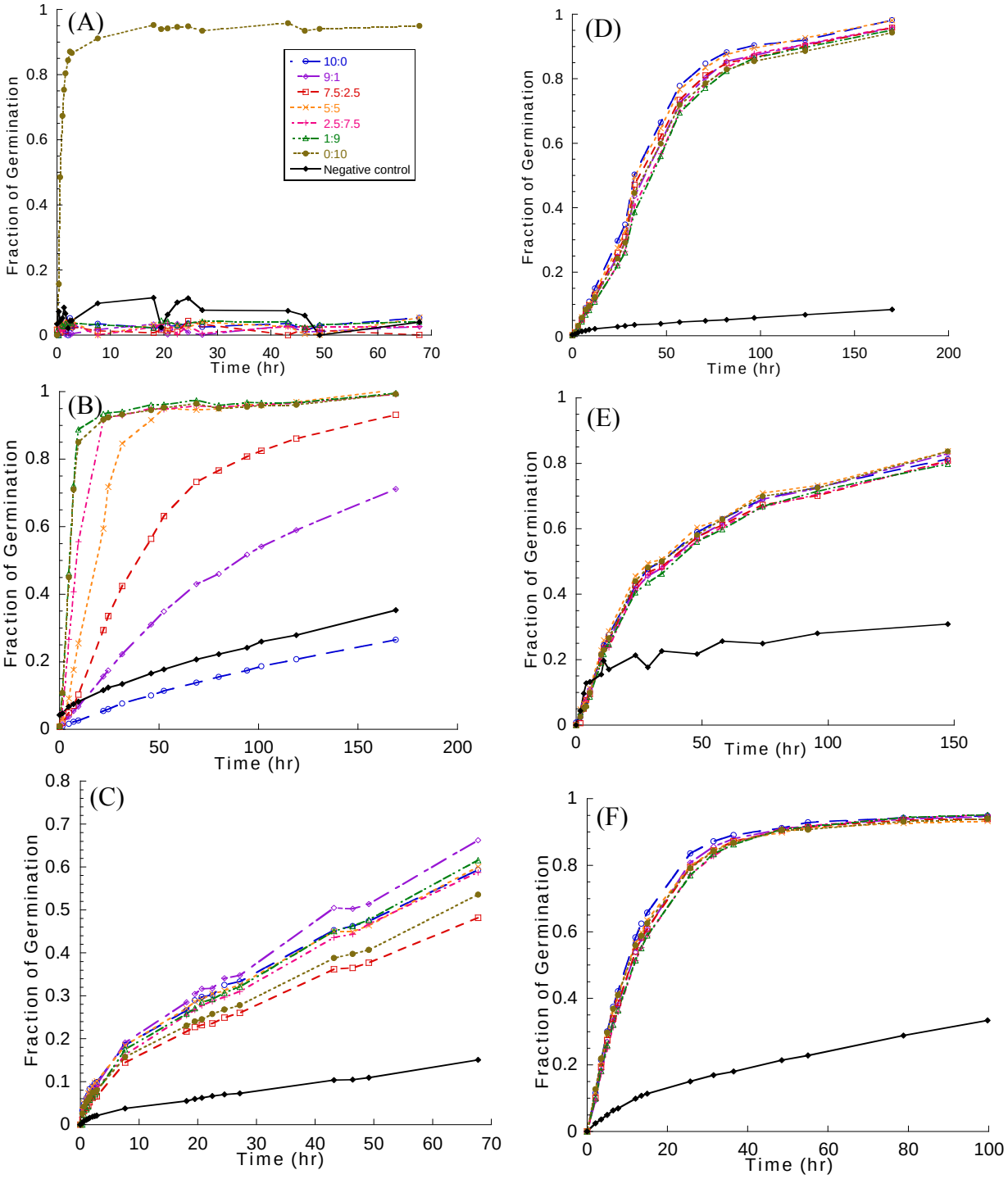

Figure 3.8 Competition experiments on spore germination of (A) *B. subtilis* from Atacama Desert, (B) *B. atrophaeus*, (C) *B. simplex* from Kilimanjaro, (D) *C. sporogenes*, (E) *C. hungatei*, and (F) *C. G5A-1* from Greenland ice cores. The ratios of D-/L-alanine

used are 10:0, 9:1, 7.5:2.5, 5:5, 2.5:7.5, 1:9 and 0:10. At any given sample, the total germinant concentration (D- plus L-alanine) is 100 mM.

CHAPTER 4: DEVELOPMENT OF A MICROSCOPIC BASED ENDOSPORE VIABILITY ASSAY TO DETECT SINGLE *CLOSTRIDIUM* SPORE GEMINATION

4.1 ABSTRACT

A microscopy based rapid endospore viability assay (Micro-EVA) has been developed to enumerate germinable *Clostridium* endospores in 15-30 minutes. Germinating *Clostridium* endospores release ~10^8 molecules of dipicolinic acid (DPA) when immobilized onto terbium ion (Tb^{3+})/D-alanine-doped agarose, which enables enumeration under UV excitation of resultant green luminescent spots with time-gated Tb^{3+}-DPA luminescence microscopy. The intensity timecourses of the luminescent spots were characteristic of the stage I germination dynamics. Micro-EVA was validated against traditional CFU cultivation over three orders of magnitude in endospore concentration, from 0 to 1000 spores/mL. Determination of the fractions of germinable *C. sporogenes* spores by Micro-EVA yielded 56.4% ± 1.5%, while only 43.0% ± 1.0% spores were culturable after 10 days incubation at room temperature. The ratio of germinable/culturable was 1.31, which was consistent with the fact that a subset of the total endospore population is germinable-but-not-culturable. With Micro-EVA, D-alanine only germinated three *Clostridium* spores, but not the five different *Bacillus* spores tested. Our results indicate that D-alanine is a potential solution to distinguish *Clostridium* spores and *Bacillus* spores.

4.2 INTRODUCTION

Bacterial endospores are dormant microbial structures that are highly resistant to chemical, physical, and radiation sterilization processes [1, 2]. They are formed when spore formers (e.g., *Bacillus* and *Clostridium*) face unfavorable growth conditions, such as starvation. Sporulation is considered one of the survival strategies of microorganisms. Once they are formed, spores can stay dormant for extended periods of time [3, 4]. The isolation of viable spore-forming bacteria from the gut of a bee trapped in Dominican amber about 25~40 million years ago, and the recovery of spores from 250-million-year-old halite crystals have been previously reported [5, 6].

The significant importance of studying endospores has been illustrated in chapter 1, which can be summarized as follow. *Bacillus cereus* is a common aerobic food-borne pathogen, and *Clostridium botulinum* and *C. perfringens* are anaerobic food-borne pathogens associated with food poisoning from canned foods [7]. *C. perfringens*, *C. difficile* and *C. tetani* are etiological agents in hospital acquired infection [8, 9]. A number of *Bacillus* species are deliberately released because they produce toxins for agricultural pest control, including *B. thuringiensis*, which accounts for approximately 90% of the bioinsecticide market [10]. The most ominous use of spore formers is the release of *B. anthracis* as a bioweapon [11-13]. Spores of sulfite-reducing clostridia (SSRC) have been considered as an indicator for the presence of pathogenic microorganisms in drinking water by European Community legislation [14]. Due to their extreme resistance to various sterilization processes that readily kill vegetative cells,

endospores are routinely employed as biological indicators to validate and monitor the effectiveness of sterilization methods employed in various industries, such as medical device, pharmaceuticals, health care, food preparation, and wastewater remediation [15].

Currently, the standard method for quantifying endospores is measuring colony forming units (CFU) after heat-shock treatment. This method requires several days of incubation, tedious anaerobic culturing technique for anaerobic spore formers, and it is only able to culture only less than 1% of environmental spores [16]. Other detection methods include DNA assay and immunoassay, which require extensive sample preparation and are labor intensive. Chapter 3 describes a spectroscopy based endospore viability assay (Spectro-EVA) to detect and quantify *Clostridium* spores *via* induced germination. Germination is triggered by various germinants, such as L-alanine/$NaHCO_3$, L-lactate or D-alanine, which causes the release of approximately 10^8 molecules of a unique biomarker, dipicolinic acid (DPA) from the spore core [17]. Spectro-EVA is based on the detection of DPA, which is detected by adding $TbCl_3$ to the spore suspension and measuring the resulting Tb^{3+}-DPA luminescence intensities [18, 19].

Despite the short detection time, Spectro-EVA has its limit. For example, it has to be conducted in liquid suspension, and it is sensitive to other chemical interferences. The limit of detection for Spectro-EVA is about 1000 spores/mL. To study the endospores from various environmental samples, a much lower detection limit is desired.

Here we report details of a rapid microscopy based endospore viability assay (Micro-EVA) in which *C. sporogenes* endospores were immobilized on terbium ion (Tb^{3+})/D-alanine-doped agarose (Figure 4.1). D-alanine serves to trigger germination, during which DPA is released from endospores. The Tb^{3+} subsequently binds DPA, resulting in green luminescent spots under UV excitation in a microscope field of view, which were enumerated as germinable endospores using time-gated Tb^{3+}-DPA luminescence microscopy (i.e., Micro-EVA). We validate Micro-EVA against culturing as a method for rapid endospore viability assessment. We also applied Micro-EVA to distinguish *Clostridium* spores from their aerobic counterparts.

4.3 MATERIALS AND METHODS

Materials

Deionized water (18.2 MΩ/cm) was obtained from an ultrafilter system (Water Pro PS, LabConco, Kansas City, MO, USA). Terbium (III) chloride hexahydrate-(99.999%), dipicolinic acid (2,6-pyridinedicarboxylic acid, DPA, 99%), L-alanine and D-alanine were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Agarose for culturing experiments was obtained from MacConnell Research (San Diego, CA). Agarose for Micro-EVA experiments was obtained from Invitrogen (Carlbad, CA). *C. sporogenes*, ATCC 7955 and *Clostridium hungatei* (ATCC No.700212) from American Type Culture Collection, (Manassas, VA, USA) was obtained as freeze-dried pellets. *B. atrophaeus* (ATCC 9372) and *B. cereus* endospores were purchased from Raven Biological Laboratories.

Endospore production and purification

C. sporogenes and *C. hungatei* were revived from frozen, dry pellets in a small volume (5~6 mL) of growth media at the optimal growth temperatures of 37 °C and 30 °C, respectively. The growth media for *C. sporogenes* and *C. hungatei* was reinforced clostridial medium (RCM) and ATCC 2135 broth (GS-2CB medium), respectively. Incubation commenced under strict anaerobic conditions with 100% N_2 headspace. The culture was transferred to fresh medium after development of visible turbidity. The production and purification of various *Clostridium* spores was performed using a protocol developed specifically for *Clostridium* species [20]. The spore suspension contained less than 1% vegetative cell material as determined by phase contrast microscopy.

Bacillus spores were obtained by applying the protocol described as follow: *Bacillus* vegetative cells were grown on TSA and inoculated onto a sporulation medium after reaching exponential growth phase. The sporulation medium contained 1.6% nutrient broth, 1.6% agar, 0.2% KCl, 0.05% $MgSO_4$, 1 mM $Ca(NO_3)_2$, 100 µM $MnCl_2{\cdot}4H_2O$, 1 µM $FeSO_4$ and 0.1% glucose (pH 7.0) [4]. After incubation at 37 °C for 1 week, cells were suspended into sterile deionized water. With phase contrast microscopy, 95% of the cells formed endospores free of sporangia. Endospores were harvested and separated from vegetative cells and debris by centrifugation at 6,300 g, washing 10 times and sonication (25 kHz) for 5 min. The endospore suspension was incubated in lysozyme (0.2 mg/mL) and trypsin (0.1 mg/mL) at 30 °C with constant stirring overnight to lyse and degrade vegetative cells. Endospores were purified by 8 cycles of centrifugation (6,300 g)

and washed with sterile deionized water until >99.9% of the cells were fully refractile with no noticeable cellular debris.

Both *Clostridium* and *Bacillus* spore suspensions were stored at 4 °C in the dark before use. Total spore concentrations were determined using a Petroff-Hausser hemocytometer and CFU concentrations were determined using spread plating in triplicate measurements.

Sample preparation for Micro-EVA experiments

Endospore suspension with known concentration was filtered onto a 1.5 mm^2 spot on a 0.2 μm polycarbonate membrane filter (Whatman, Florham Park, NJ) using a 96-well micro-sample filtration manifold (Schleicher & Schuell, Keene, NH). To ensure that the endospore surface density was optimal for a given initial endospore concentration, each microscopic field of view contains less than 300 endospores. Endospores concentrated on the filter were transferred to a ~0.5 mm thick, 9 mm diameter slab of 1.5% agarose substrate containing 5 mM $TbCl_3$ and 100 mM L-alanine (or D-alanine) mounted in a silicone isolator (Molecular Probes, Eugene, OR) on a quartz microscope slide. After endospore transfer, the agarose surface was covered with a piece of 0.2 mm thick polydimethylsiloxane (PDMS).

Polydimethylsiloxane (PDMS) was prepared by mixing the polymer base and curing agent (Sylgard, Dow Corning) in 10 to 1 ratio. After degassing, the mixture was cast over

a 0.2-mm thick stainless steel mold and cured in an oven for 2 hours at 65 °C. Agarose, silicone isolator and PDMS were autoclaved at 121 °C for 15 minutes before use. A piece of PDMS was peeled off and attached on top of an endospore-laden agarose surface for sealing.

The Micro-EVA instrument

The instrument consists of a time-gated camera (Photonics Research Systems, Salford, UK) mounted on a Nikon SMZ800 stereoscopic microscope (large working distance for xenon lamp), a xenon flashlamp (Perkin-Elmer, Waltham, MA) mounted at 45 degrees with respect to the sample, and a temperature controlled microscope slide holder (Thermal). The slide holder enabled endospores to germinate at 37 °C. The CCD camera has a resolution of 752 × 582 pixels at 14 bits with a chip size of 2/3 inch. The camera has 50% sensitivity between 430 and 730 nm, with peak sensitivity at 550 nm. It was Peltier-cooled to 40 °C below ambient temperature and was synchronized to the xenon lamp *via* TTL pulses (300 Hz with tail time up to 50 μs). A highpass filter (03FCG067, Melles Griot) centered at 500 nm was placed along the light path on the emission side before reaching the microscope objective. We collected time stacks of time-gated images by real-time streaming with a delay of 100 μs and exposure time of 5 s in each frame.

Endospore germination and germinable endospore assignment

Endospore germination on agarose surface followed the reported micro-germination dynamics [21]. DPA released from single endospores manifested as individual bright

spots in 30 minutes under time-gated microscopy due to local formation of Tb^{3+}-DPA. Assignment of germinable endospores was made based on intensity, size and germination timecourse. Adaptive thresholding was applied to segment pixels that were 20% brighter than the background with a characteristic rising intensity. Each bright spot must exhibit a continuous rising intensity over the course of germination in order to register an endospore count. This criterion eliminated false positives by not counting sporadic bright spots and long-lived luminescent interference. The 8-connected adjacent pixels were analyzed to screen for endospore clumps. The number of endospores present was calculated by dividing the squared sum of neighboring pixel brightness by the mean brightness of an individual endospore determined empirically. This was done in a recursive way until all of the pixels were counted and marked.

Phase contrast microscopic enumeration to determine spore concentrations

Aliquots (5 μL) of spore suspension were placed in a Petroff-Hausser counting chamber (Model 3900, Hausser Scientific, Horsham, PA), and spores were observed at 400× magnification using a phase contrast microscope (Nikon Eclipse 80i, AG Heinze Co, Lake Forest, CA) mounted with a digital camera (Nikon Digital Sight DS-5M). The smallest squares in the counting chamber are 0.05 mm × 0.05 mm, of which 80 were analyzed to obtain the average spore count per square. To obtain statistically significant counts, the spore concentration was held above 10^7 spores/mL, which resulted in at least 10 cells per sixteen squares.

Spore culturability

To determine the endospore culturability, heat-shock treatment at 80 °C for 15 minutes was applied to a purified spore suspension with a known concentration that was first determined by microscopy as described above. A series of dilutions were made to reach an expected concentration range of 0 to 1000 spores/mL. For higher concentration (1000, 333 spores/mL), one hundred µL aliquots from each dilution were plated in triplicate on solidified Reinforced Clostridial Medium (15 g agar per liter). For lower concentration (below 100 spores/mL), 1 mL aliquots from each dilution were plated in triplicate on the same solidified medium. Plates were incubated in a vinyl anaerobic chamber (Type C, Coy Laboratory Products Inc., Grass Lake, Michigan) at room temperature for 10 days. Colonies were counted and the average number was designated as colony forming units (CFU) per volume of original sample. The culturability was determined as the percentage of spores capable of forming colonies.

Spectroscopy

Tb^{3+}-DPA luminescence excitation spectra (λ_{ex}=250-360 nm, λ_{em}=544 nm) and emission spectra (λ_{ex}=278 nm, λ_{em}=450-650 nm) were recorded with a fluorimeter (Model FL-1089, Jobin Yvon, Edison, NJ, USA) consisting of a 500-W Xe-lamp for excitation, two double-monochromators set at 4 nm bandpass, and a Pelletier-cooled photomultiplier tube (Model R928, Products for Research Inc, Danvers, MA, USA). A 500 nm cut-off filter (Omega Filters, Brattleboro, VT, USA) was placed at the entrance of the emission monochromator to prevent second-order diffraction. Endospores immobilized on agarose were measured in front face configuration.

4.4 RESULTS

Germination time course for single *Clostridium* spore

Spores of *C. sporogenes* were used as the model microorganism for this study. Germination of spores on Tb^{3+}/germinant (L-alanine or D-alanie) doped agarose was confirmed by the detection of DPA release *via* excitation spectroscopy using a Fluorolog fluorimeter in a front face configuration (Figure 4.2). Figure 4.3 A showed the Micro-EVA time-lapse images of a germinating *C. sporogenes* spore. DPA release was observed with micor EVA *via* DPA complexation with the Tb^{3+}-doped into the agarose matrix, which can be visualized as a bright spot around the spore. The timecourse showed the increase of intensity during germination as DPA molecules were released, and the intensity reached a plateau after 15 minutes, indicating the completion of germination. Figure 4.3 B showed single germinated spores under Micro-EVA in one field of view. In combination, these data established that Micro-EVA can be used to observe single spore germination on agarose.

Validation of Micro-EVA against CFU culturing

To validate Micro-EVA, we performed parallel germination and culturing experiments over three orders of magnitude in endospore concentration, from 0 to 1000 spores/mL. Figure 4.4 shows the germinable endospore concentrations measured with Micro-EVA, and culturable endospore concentrations measured with plate counting plotted against total endospore concentrations as determined with phase contrast microscopy. Sterile samples did not yield false positive counts, which enabled us to achieve the ultimate

sensitivity of one germinable endospore per Micro-EVA field of view. Of the total endospore concentrations, Micro-EVA revealed that 56.4% ± 1.5% of the population were germinable within 60 minutes and CFU culturing determined that 43.0% ± 1.0% were culturable within 10 days incubation. The ratio of germinable/culturable was 1.31, which is consistent with the fact that a subset of the total endospore population is germinable-but-not-culturable.

Discrimination of *Clostridium* from *Bacillus* spores

D-alanine was used as the germinant to distinguish *Clostridium* spores from *Bacillus* spores. D-alanine has been reported as a germination inhibitor for various *Bacillus* spores, however, its effect on *Clostridium* spore germination has not been extensively studied. We tested the effect of D-alanine on germination with various *Clostridium* and *Bacillus* spores with Spectro-EVA, and found that in liquid suspension, D-alanine germinated all *Clostridium* spores tested, while it inhibited the germination of all but one *Bacillus* spores, *B. simplex* isolated from Kilimanjaro (Chapter 3). This indicated that D-alanine may be a potential solution to distinguish *Clostridium* spores and *Bacillus* spores.

We conducted Micro-EVA experiment with D-alanine as the only germinant on five different *Bacillus* spores and three *Clostridium* spores. The *Bacillus* spores used in this study are: *B. subtilis* isolated from Atacama Desert, *B. simplex* isolated from Kilimanjaro, *B. atrophaeus* and *B. cereus* from ATCC, *B. longisporus* isolated from JPL soil samples. The *Clostridium* spores used in this study are *C. sporogenes* and *C. hungatei* from ATCC,

and *Clostridium G5A-1* isolate from Greenland ice core. With all the *Bacillus* spores tested, none of them germinated with D-alanine as the sole germinant on agarose with Micro-EVA, including the Kilimanjaro strain that germinated by D-alanine in liquid suspension with Spectro-EVA. All three *Clostridium* spores germinated by D-alanine, indicated as bright spots under UV light on agarose after 1 hour germination at 37 °C (Figure 4.5).

4.5 DISCUSSION

We have developed a microscopy based Endospore Viability Assay (Micro-EVA) to detect anaerobic spores, and to measure the germinable spores in a solid phase. Micro-EVA has also been validated with respect to traditional CFU cultivation. Micro-EVA yielded 56.4% ± 1.5% germinable spores with 60 minutes, while CFU revealed 43.0% ± 1.0% culturable spores for the same suspension after 10 days incubation at room temperature. The difference can be explained as only a subset of germinable spores is culturable. Micro-EVA measurements probe DPA release during stage 1 germination, while CFU measurements probe the cell division, which is the last stage of the germination and outgrowth process, and requires proper growth conditions (e.g., enough nutrient, and anaerobic environment for *Clostridium* spores). Micro-EVA proved to be a faster method to assess spore viability, as germinability has been proposed as the indicator of spore viability [17, 22].

In Micro-EVA experiments, individual germinable spores are counted in a microscope field of view after germination commences. As a spore germinates, ~10^8 DPA molecules are released into the immediate area surrounding the spore. DPA then combines with Tb^{3+} in the agarose matrix to form the Tb^{3+}-DPA luminescence halos under UV excitation. The germinating endospores manifest as bright spots that grow in intensity over a period of 3-5 minutes, and are enumerated in a microscope field of view. The characteristic germination timecourse allows unambiguous assignments of germinable spores.

In chapter 3, we reported a related method where germinating spores were enumerated in bulk suspension by luminescence spectroscopy (i.e., Spectro-EVA), where Tb^{3+}-DPA luminescence intensities were tabulated against a *C. sporogenes* spore calibration curve [17]. The Micro-EVA approach is superior to Spectro-EVA because Micro-EVA is capable of enumerating single spores while the limit of detection of Spectro-EVA is 1000 spores/mL. This advantage is gained because in Micro-EVA experiments the mM DPA halos surrounding single germinated endospores are readily imaged with high contrast, whereas germination of single endospores in bulk suspension (~1 mL) gives rise to mere fM DPA concentrations, which are far below LOD for Spectro-EVA. With Micro-EVA, we take advantage of the long luminescent lifetime (τ = 0.5 to ~2 ms) of Tb^{3+}-DPA [23], enabling the use of time gating to effectively remove background fluorescence (i.e., interferent fluorophores with nanosecond lifetimes). Time gating eliminates potential false positive causing features and renders the image background dark. Elimination of this background enables a striking increase in image contrast and detection sensitivity

even for the most challenging environmental extracts, such as soil samples. This will be illustrated in chapter 5.

The difference between *Bacillus* and *Clostridium* spores has received increasing attention recently due to the advance of molecular technology. It has been reported that the difference of these two genera lies in the initiation of sporulation [24]. However, the study to distinguish spores from these two genera has not been reported. Here, we investigate this issue by studying the response of *Clostridium* and *Bacillus* spores to different germinants. Our preliminary results show that D-alanine, D-serine and lactic acid inhibit *Bacillus* spores germination, while all chemicals facilitate the germination of *Clostridium* spores (data not shown). We picked D-alanine for further study. Our results show that on solid phase with Micro-EVA, D-alanine does not germinate all *Bacillus* spores tested, while it germinates all *Clostridium* spores. We have not investigated in why spores of *B. simplex* from Kilimanjoro germinate in liquid solution (Spectro-EVA) with D-alanine as the sole germinant but not on solid phase (Micro-EVA). Our results support that D-alanine can indeed distinguish *Clostridium* spores from *Bacillus* spores on solid phase with Micro-EVA. In fact, D-alanine has been reported as a germination inhibitor for various *Bacillus* spores by competing with L-alanine for the active site of dehydrogenase [25-27]. However, D-alanine does not have any inhibitory effect on the *Clostridium* spore germination, despite the similarity between these two genera. Our results provide insight to understand the germination mechanism for *Clostridium* spores.

Micro-EVA is not only much more rapid than culture dependent methods (30 minutes vs. 3-10 days), but the simple chemistry, instrumentation, and image analyses are all amenable for automation. Automated viability assessment of endospores will have the potential to find application in many industries where microbial inactivation needs to be monitored and assured. Potential applications of Micro-EVA for *Clostridium* endospores include evaluating the efficacy of sterilization regimens for food, wastewater, and medical equipment. To test a given sterilization regimen, we envision that surrogate microorganisms (e.g., *C. sporogenes* spores) would be applied to the appropriate matrix (i.e., food, wastewater, medical equipment), and after the sterilization process is complete, the matrix is extracted, and the extract analyzed with the Micro-EVA. By comparing the percentage of germinable spores before and after a specific sterilization treatment, we are able to evaluate if the treatment is efficient or not. This is particularly important for the new sterilization technologies, since Micro-EVA can potentially monitor the in situ inactivation dynamics, which is indispensable for identifying the inactivation regime and validating the new technology. Ultimately, with implementation of automated sample handling and analysis, we envision that Micro-EVA will enable online monitoring of sterilization processes for various sterilization processing facilities.

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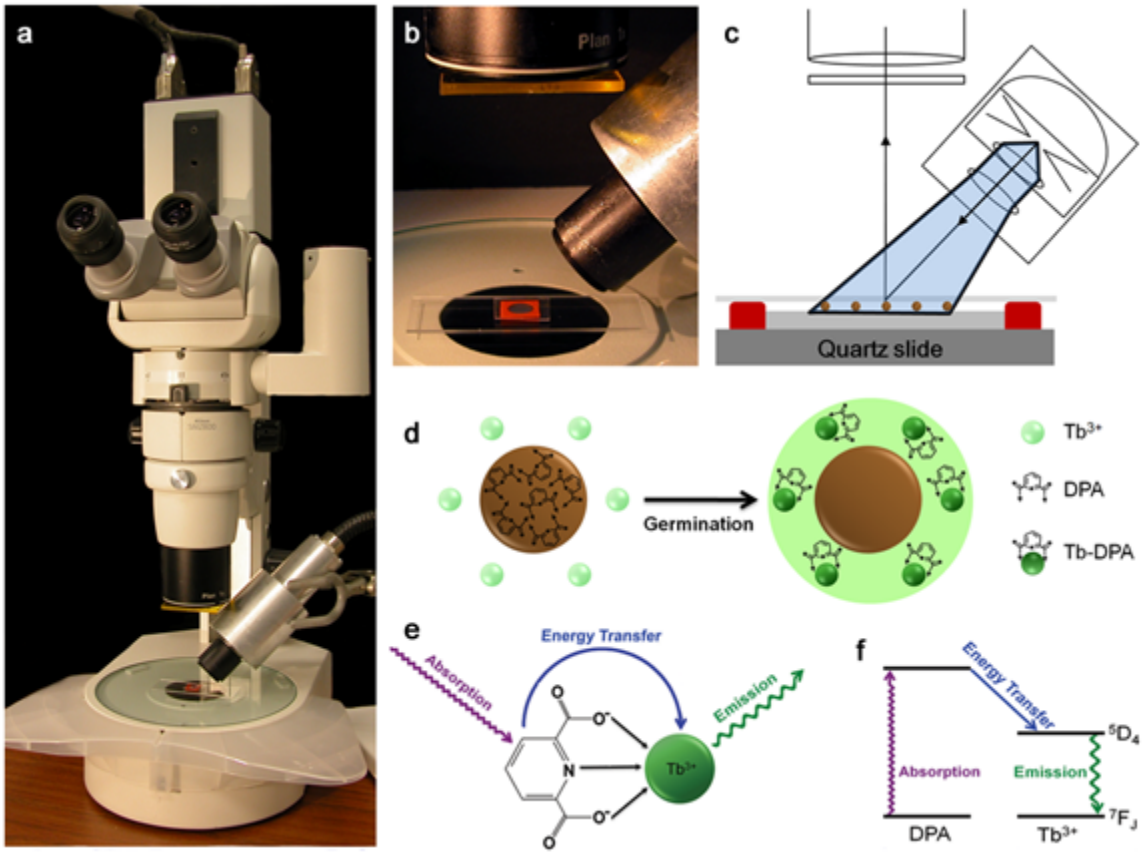


Figure 4.1 (a) Configuration of the Micro-EVA instrument used in this investigation, consisting of a stereomicroscope mounted with a time-gated camera and a xenon flash lamp for UV excitation. **(b)** Sample well on quartz microscope slide containing Tb^{3+}/D-alanine doped agarose. **(c)** Schematic representation of the sample slide, consisting of a quartz slide on which Tb^{3+}/D-alanine doped agarose is confined by red rubber gasket well. Endospores (brown circles) are inoculated onto agarose substrate and subsequently covered with a thin layer of PDMS. **(d)** Inoculated endospores germinate due to D-alanine, causing the release of ~10^8 molecules of DPA and subsequent formation of highly luminescent Tb^{3+}-DPA complexes that appear as discrete bright spots in the microscope field of view. **(e)** Absorption-energy transfer-emission photophysics of the Tb^{3+}-DPA luminescence assay. DPA acts as a light harvester that transfers excitation

energy to luminescent terbium ion. **(f)** Energy (Jablonski) diagram of the Tb^{3+}-DPA photophysics.

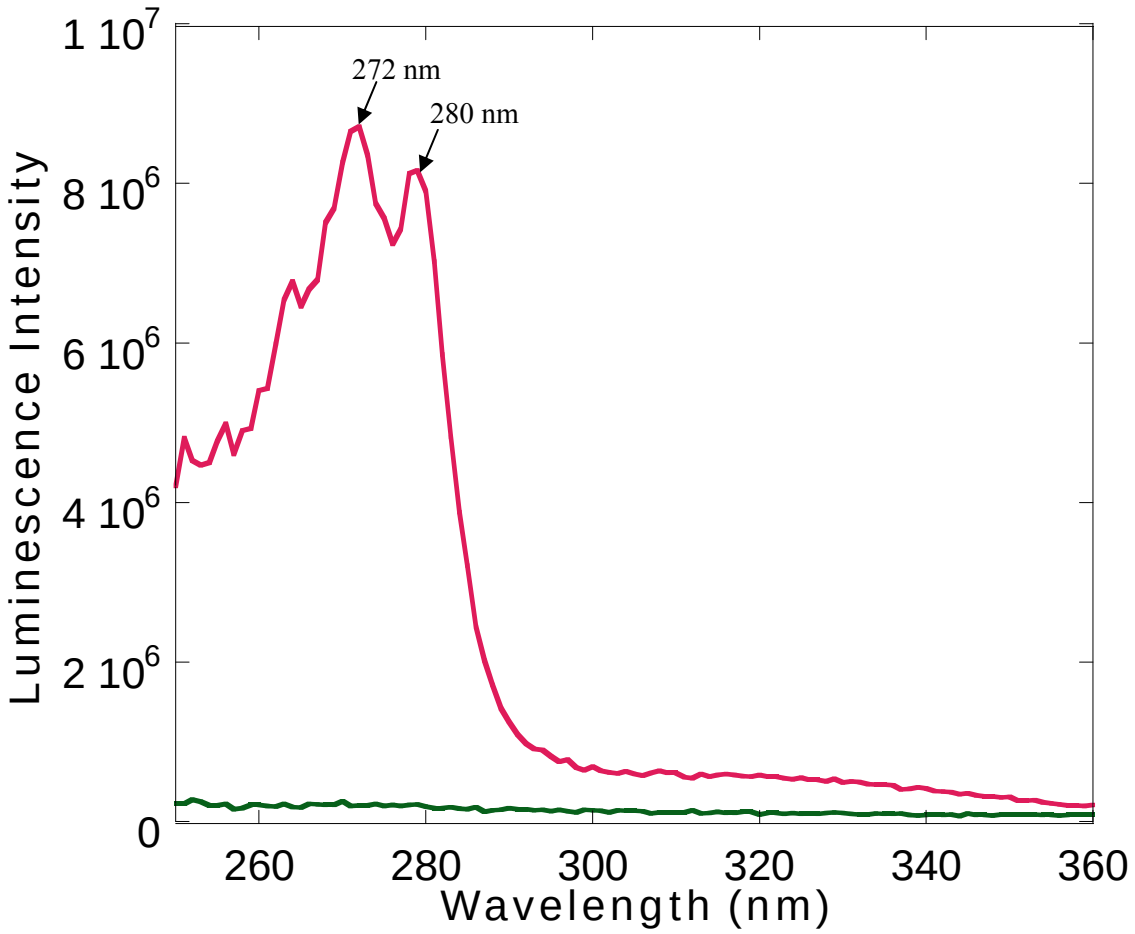
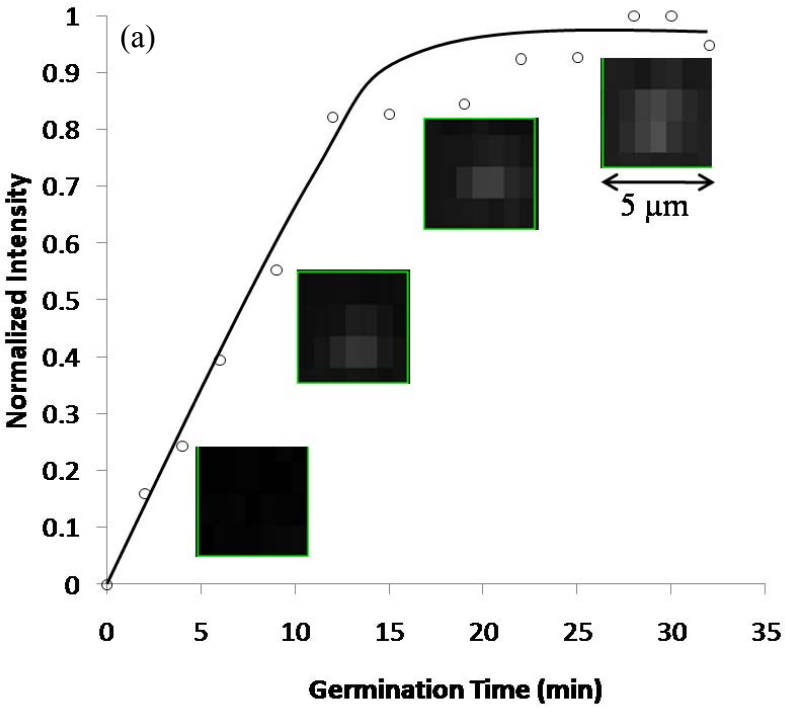


Figure 4.2 Excitation spectrum (λ_{em} = 545 nm) of the agarose substrate of Micro-EVA when 10^8 *C. sporogenes* endospores were inoculated, taken with a Fluorolog-3 fluorimeter at a front-face configuration. The dual excitation peaks (λ_{ex} = 272, 280 nm) indicated the presence of Tb^{3+}-DPA released from germinating spores. Red plot represents germinating endospores on agarose and the green plot shows the agarose substrate.



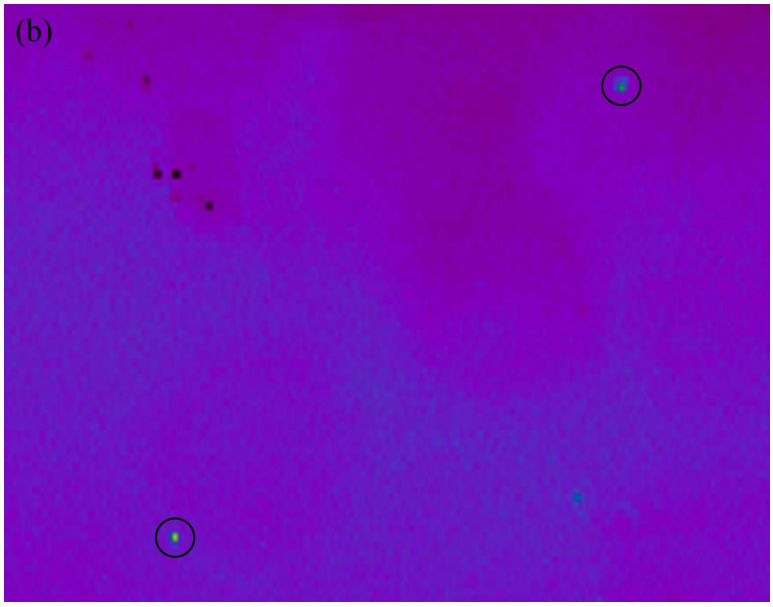


Figure 4.3 (a) Germination timecourses of single *C. sporogenes* spores at 37 °C monitored by Tb^{3+}-DPA luminescence using Micro-EVA. **(b)** Two single germinated spores under Micro-EVA in one field of view. Purple color is the background intensity; germinated spores are luminescent as green spots under UV excitation.

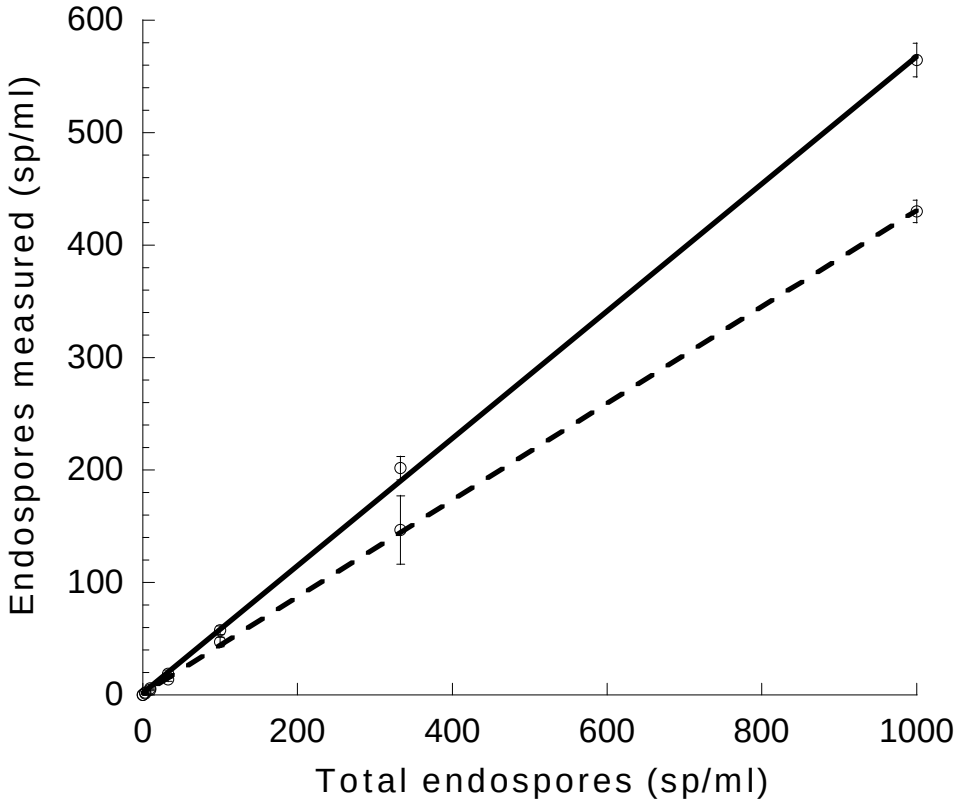
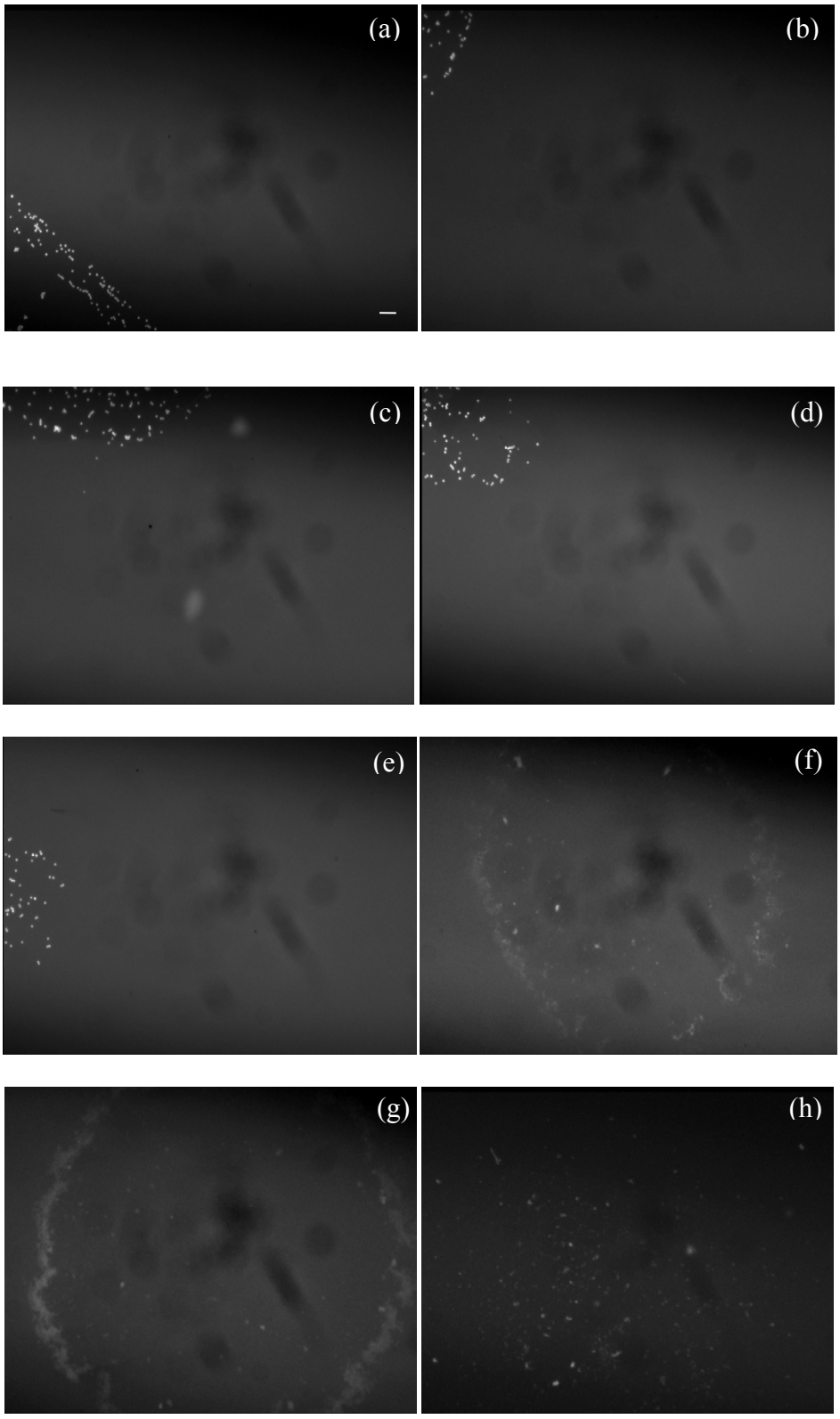


Figure 4.4 Endospore concentration dependence measured with Micro-EVA (solid line) and CFU cultivation (dashed line) versus total endospore concentrations as determined by phase contrast microscopy.


(a)
(b)
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(g)
(h)

Figure 4.5 Endospores inoculated on Tb^{3+}/D-alanine-doped agarose. **(a)** *B. subtilis* from Atacama Desert, **(b)** *B. simplex* from Kilimanjaro, **(c)** *B. atrophaeus* (ATCC 9372), **(d)** *B. cereus*, **(e)** *B. longisporus* from JPL soil, **(f)** *C. hungatei* (ATCC 700212), **(g)** *Clostridium G5A-1* from Greenland ice core, **(h)** *C. sporogenes* (ATCC 7955). The bright spots on images of *Bacillus* spores are Tb microspheres, which serve as internal standard to facilitate locating the focal plane. Scale bar: 100 μm.

CHAPTER 5: APPLICATION OF MICRO-EVA TO DETECT *CLOSTRIDIUM* SPORES FROM GREENLAND ICE CORE AND ATACAMA DESERT

5.1 ABSTRACT

Understanding the boundary conditions for survival of microorganisms in extreme environments will aid the NASA effort to search for life on Mars and prevent forward contamination from spacecraft before launching. We investigated the limit and longevity of life in two Mars analogs on Earth, Greenland ice core and Atacama Desert. While the spectroscopy based endospore viability assay (Spectro-EVA) failed to quantify germinable endospores in most environmental samples, the microscopy based endospore viability assay (Micro-EVA) has been successfully employed to detect and quantify viable *Clostridium* spores from these extreme environments. Micro-EVA is based on the detection of a unique biomarker of endospores, DPA, *via* terbium ion (Tb^{3+}-DPA) luminescence. DPA is released during D-alanine induced germination, an indicator for spore viability. Greenland ice core samples yielded 1 to 2 germinable *Clostridium* spores per milliliter of melt ice water after 2-hour germination, and our culture based study also showed that 10 different *Clostridium* species are revived from the ice core samples with age up to 60,000 years old. The Atacama Desert samples yielded 66 to 157 germinable *Clostridium* spores/g soil after 90-minute germination. Our preliminary findings support the hypothesis that the higher water activity of the environmental sample, the more germinable spores are detected from it. In conclusion, Micro-EVA has been successfully

applied to assess germinability of *Clostridium* spores in extreme environments. Studying these extreme habitats on Earth gleans invaluable information on the search for extinct or extant life on the dry and icy biosphere, Mars.

5.2 INTRODUCTION

The first astrobiological experiment conducted by the Viking missions more than three decades ago concluded that life had little chance of developing on Mars due to the harsh conditions detected on the surface of the planet: intense UV radiation, generation of oxidant compounds, absence of water and low temperatures [1]. However, in recent years, the discovery of extremophiles, organisms that can thrive under conditions formerly considered unsuitable for life, such as extreme temperatures, pressures, pH, opens the window on possibilities that life exists in these extreme conditions [2, 3]. There are various places on Earth considered as Mars analogs due to their extreme physical and chemical conditions [4], such as the Antarctic ice sheets and underground lakes, Atacama Desert [2]. Research on life in these extreme environments sheds light on several important exobiological questions in the past: What are the environmental limits for life? How long can microorganisms survive? Is there life beyond Earth? Can life travel from one planet to another?

Approximately 75% of Earth's biosphere is cold and covered by oceans at temperatures below 5°C [5]. More than 70% of Earth's fresh water occurs as ice and a large portion of the soil ecosystem exists as permafrost. These permanently frigid habitats are unique repositories of microbes frozen at various points in the geologic past. A diversity of

viable microorganisms, including fungi, bacteria and archaea have been isolated from glacial ice up to 400,000 years old and permafrost up to 3 million years old, originating from many geographical locales [6-11]. The extent to which organisms can survive extended periods in metabolic inactive form in ice cores is one of the key questions in the study of life in extreme environments. Ice cores, as an environment with low and more or less constant temperature, is the region where remnants of life or even dormant forms of life would be preserved for the longest time. The ice sheets of Greenland are considered suitable sites for researchers to study longevity of microbial cells at low temperatures [12]. GISP2 (Greenland Ice Sheet Project 2) is an international collaborative project that lasted from 1993 to 1998 to drill and collect ice cores from different depths along the 3053.44 m ice, which corresponding to samples aged ~110,000 years old. Only a few studies have been carried out on bacterial communities within ice cores, however, the population of bacterial spores in these communities has never been directly assessed [13-16]. We will address this question in this chapter.

As one of the oldest and driest desert on Earth, Atacama Desert in Northern Chile is considered one of the Mars analogs on Earth. It is a cold desert with a hyper arid habitat [2]. The average annual air temperature is -5 °C to 45 °C, depending on elevation and distance from the sea. The annual precipitation is less than 1 mm. Some of the weather stations in the Atacama Desert have never received rainfall records. The cold water brought from Antarctica along the Chilean coast creates a phenomenon called the temperature inversion in which cold air is below a ceiling of warm air, preventing water evaporation and formation of clouds. Also, the desert is in the rain shadow of the Andes

on the west and Chilean Coast Range on the north, within the dry subtropical climate belt [17, 18]. Owing to the combined effects of these physical factors, the Atacama Desert has been devoid of water for over 150 million years [19-21], and it has been reported to contain near sterile soils with only trace organic carbon and nitrogen content, and these conditions have existed for at least several millions years [22]. The upper soil layers are dry and weathered. The soil environment precludes any downward infiltration. For this reason, the possibility of penetration of model microorganisms into the soil subsurface is precluded, making it a very good model to assess the dry limit of life, effect of water on microbial abundance and preservation of organic material along a depth transect of soil. It is also one of the best repositories for life on Earth because of minimal biological degradation of dead cell debris. Our research group went on an expedition to the Atacama Desert in 2007, and collected samples from the most arid zone, the Yungay area, and a depth transect in a soil pit 80~220 cm underneath the surface soil. Samples from two sites in the Yungay area were collected: Site A and Site E. Site A is the location of a past effort to extract DNA and cover culturable microorganisms from the soils of this region [22], which did not yield detectable concentrations of DNA and low culturable cell counts. Site E is 5 km northeast of Site A, and is the location of prior investigations that focused on soil chemistry, identification of cultured isolates and DNA extraction [23, 24].

Formation of the remarkably resistant and dormant endospores in genera such as *Bacillus* and *Clostridium* is observed as a response to adverse conditions, such as nutrient depletion and low temperature [25]. Due to their resistance to various extreme stresses, endospores are one of the life forms that are most likely to survive in the extreme

environments mentioned above. In fact, a six-year space study also showed that spores of *Bacillus subtilis* survived after exposure to space environments such as high vacuum and radiation [26]. If endospores were present in an early warm and wet Martian environment, they may currently be surviving in the subsurface ice/permafrost. Assessment of viable endospores from Greenland Ice Core and Atacama Desert provides insight on the limit and longevity of life, and the potential of extraterrestrial life. We have described two endospore viability assays, Spectro-EVA and Micro-EVA, which are based on the detection of the release of a unique biomarker, dipicolinic acid (DPA) upon induced germination. In this chapter, we applied of these assays to assess of viable *Clostridium* spores from these two Mars analogs. As comparison, culture-based and DNA extraction studies have been also included.

5.3 MATERIALS AND METHODS

Materials

Deionized water (18.2 MΩ/cm) was obtained from an ultrafilter system (Water Pro PS, LabConco, Kansas City, MO). Terbium (III) chloride hexahydrate-(99.999%), dipicolinic acid (99%) (2,6-pyridinedicarboxylic acid, DPA), and D-alanine were obtained from Sigma-Aldrich (Milwaukee, WI), and were used without further purification. Sodium pyrophosphate was purchased from Mallinckrodt (Paris, KY). Agarose for culturing experiments was obtained from MacConnell Research (San Diego, CA). Agarose for Micro-EVA experiments was obtained from Invitrogen (Carlbad, CA). All utensils were either prepackaged one-use only or glassware was kilned at 500 °C for 4 hours for

sterilization and removal of biomolecules such as DNA, RNA and DPA. All media and utensils were pre-chilled to 4 °C before use for the Greenland ice core experiments.

GISP2 ice core handling

The ice core was received as a gift from the Greenland Ice Sheet Project (GISP 2) and corresponded to 14 depths from 158 to 2804 m. The GISP2 ice cores, arranged in ascending depth order (MCA.02 # 158, depth 157.45-157.70 m, age 600 years; MCA.02 # 480, depth 480.15-480.40 m, age 2000 years; MCA.02 # 835, depth 834.10-834.35 m, age 4000 years; MCA.02 # 1566, depth 1565.75-1566.00 m, age 10,000 years; MCA.02 #1923, depth 1922-1922.25 m, age 20,000 years; MCA.02 #2096, depth 2095.75-2096.00 m, age 30,000 years; MCA.02 #2253, depth 2252.10-2252.35 m, age 40,000 years; MCA.02 #2429, depth 2428.00-2428.25 m, age 50,000 years; MCA.02 #2517, depth 2516.75-2517.00 m, age 60,000 years; MCA.02 #2593, depth 2592.31-2592.56 m, age 70,000 years; MCA.02 #2657, depth 2656.20-2656.45 m, age 80,000 years; MCA.02 #2703, depth 2702.11-2702.36 m, age 90,000 years; MCA.02 #2746, depth 2745.65-2745.90 m, age 100,000 years; MCA.02 #2804, depth 2803.65-2803.90 m, age 110,000 years), were obtained from the National Ice Core Laboratory (NICL) and stored at -80 °C. In preparation for decontamination, the ice core temperature was ramped to 0 °C via storage first at -25 °C for eight hours, followed by two hours at 0 °C. Temperature equilibration of the ice core was necessary to prevent the decontamination solutions from freezing onto the outside of the ice core.

Decontamination was carried out under aseptic conditions, as illustrated in figure 5.1. The core sections were placed on an aluminum block (pre-chilled at -80 °C freezer and disinfected by bleach). A heat-sterilized saw was used to cut the core into half. One half underwent the decontamination process and the remaining portion was stored at -80 °C for future processing. A beaker containing 1600 mL of cold (4 °C) 6.25% sodium hypochlorite (NaOCl) solution and 3 beakers containing 1600 mL of cold (4 °C) sterile distilled water (18.2 MΩ, < 1 ppb TOC, DNase-free and RNase-free) were prepared. The ice core was submerged in the NaOCl solution for 10 s and then rinsed with sterile water 3 times, each with 10 s, and finally placed in another beaker for melting aseptically at room temperature inside a biohazard cabinet hood (Sterilgard III Advance, The Baker Company, Sanford, ME). The decontamination procedure has been validated using a synthetic ice core sections (a cylinder 5 cm in diameter and 15 cm in length), consisting of frozen suspensions of 10^4 *B. atrophaeus* endospores in the interior, and 10^5 *B. megaterium* endospores smeared on the exterior surfaces.

Before melting, 1/10 of the meltwater was reserved for culturing. The remaining meltwater was vacuum filtered through a 45 mm 0.1 μm membrane filter (Nucleopre Track-etch membrane, Sterlitech Corporation, Kent, WA) backed with a 0.45 μm Supor backing filter. The meltwater was analyzed with 24 hours by culturing. The filter(s) was/were resuspended in 4.4 mL of the original cold filtrate (or cold sterile water for the first three pieces ice core, depth 158 m, 480 m and 835 m). The suspension and filter(s) were vortexed for 5 minutes, with 1-minute chilling increment in between to prevent cells from overheating. The membrane filter(s) was/were removed from the suspension and

stored in -20 °C freezer for clone library analysis. The concentrated suspension was used for subsequent endospore viability assays.

Ice core analysis using Spectro-EVA

For the older ice cores (age 10,000 years old and above), the ice core suspension was prepared in 300 µL containing 100 mM L-alanine, 10 mM $NaHCO_3$ and 1 µM $TbCl_3$ at pH 6.5 to induce germination of *Clostridium* spores, and for remaining ice cores, ice core suspension was prepared in 250 µL containing 100 mM D-alanine to induce *Clostridium* spores germination. The ice core suspensions were incubated at 37 °C for 48 hours to let germination complete. After germination, ice core suspension was transferred to an eppendorf tube and autoclaved at 134 °C for 45 minutes to release DPA from ungerminated spores. Before and after germination, and after autoclaving, Tb^{3+}-DPA luminescence excitation (λ_{ex} = 250 – 360 nm, λ_{em} = 544 nm) and emission (λ_{ex} = 278 nm, λ_{em} = 450 - 650 nm) spectra were recorded at room temperature with a fluorimeter model FL-1089 (Jobin Yvon, Edison, NJ) consisting of a 500-W Xe-lamp for excitation, two double-monochromators set at 4-nm bandpass, and a Pelletier-cooled photomultiplier tube model R928 (Products for Research, Inc., Danvers, MA). Emission intensities were calibrated with respect to an external Tb^{3+}-DPA luminescence standard solution. Luminescence intensities were quantified by integrating the area under the 544-nm emission peak with a baseline subtraction. Quartz micro-cuvettes (Starna Cell) were used for minimal dilution factor. The aerobic version of Spectro-EVA has also been employed on all ice cores with a change in the germinant used. 100 mM L-alanine was used as a sole germinant for *Bacillus* spores.

Ice core analysis using Micro-EVA

Micro-EVA experiments were performed as previously described in chapter 4. Briefly, a time-gated microscopy technique was used to count individual endospores in a microscope field of view. The contrast in the image is generated by the formation of the long-lived and brightly luminescent Tb^{3+}-DPA complex as DPA is released during the germination process. DPA release is observed as a spreading halo originated from each endospore during germination, where DPA molecules bind with Tb^{3+} that has been added into the surrounding matrix. The germinating endospores manifest as bright spots in the field of view that grow in intensity over a period of 3-5 minutes.

A 100 μL aliquot of the concentrated sample was filtered onto a 1.5 mm^2 spot on a 0.2 μm polycarbonate membrane filter (Whatman, Florham Park, NJ) using a 96-well micro-sample filtration manifold (Schleicher & Schuell, Keene, NH). The sample was transferred aseptically to 1.5% agarose doped with 5 mM $TbCl_3$ and 100 mM D-alanine, contained in a 9 mm silicone well (Molecular Probes, Eugene, OR) on a quartz slide. The sample was transferred by streaking the membrane filter across the agarose surface. The D-alanine within the agarose triggers *Clostridium* spores present in the sample to germinate and release DPA. A piece of 4.5 μm thick polydimethylsiloxane (PDMS) film was placed on top of the agarose to prevent aerial contamination, minimize water evaporation and DPA diffusion across the agarose substrate. Images were taken with a time-gated microscope, which consists of a pulse xenon flashlamp, a stereoscopic microscope (1 mm × 0.8 mm field of view at 63× magnification), mounted with a time-gated camera (Photonics Imaging System, UK). The timing of gating is governed by the

xenon flashlamp, which serves as the master oscillator at 300 Hz. After a delay of 100 μs after the lamp flash, the CCD camera begins integration of single photons. Over a germination course of 120 minutes, formation of luminescence spots could be observed under time-gated microscopy, which was indicative of the localized formation of Tb^{3+}-DPA due to DPA release during endospore germination. Background fluorescence and autofluorescence were minimized because of time gating, rendering a low intensity background. Characteristic germination timecourse allows unambiguous assignment of germinating endospores.

CFU Cultivation

Cultivation experiments were only conducted with older ice cores (age 10,000 and above). 1/10 R2A was used for the cultivation of aerobic bacteria. Two solid media, MM2 and 1/10 R2A, were used for the growth of anaerobic bacteria. Both aerobic and anaerobic cultures were incubated at two different temperatures: 7 °C and 22 °C. MM2 consists of basal mineral salts ($CaCl_2 \cdot 2H_2O$, 0.2 g/L; KH_2PO_4, 0.5 g/L; K_2HPO_4, 0.5 g/L, Na_2SO_4, 1 g/L; NH_4Cl, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.2 g/L; NaCl, 0.8 g/L), trace elements ($FeCl_2 \cdot 4H_2O$, 0.003 g/L; $CuCl_2 \cdot 2H_2O$, 2×10^{-4} g/L; $MgCl_2 \cdot 6H_2O$, 0.001 g/L; $CoCl_2 \cdot 6H_2O$, 0.0017 g/L; $ZnCl_2$, 0.001 g/L; $H_3BO_3 \cdot 2H_2O$, 10^{-4} g/L; $NiSO_4 \cdot 7H_2O$, 3×10^{-4} g/L; Na_2SeO_3, 2×10^{-4} g/L), redox indicator (resazurine, 10^{-4} g/L), reducing agent (cysteine HCl, 0.5 g/L; $Na_2S \cdot 9H_2O$, 0.3 g/L), carbonate buffer ($NaHCO_3$, 4 g/L) and various nutrients (trypticase, 1 g/L; yeast extract, 1 g/L; glucose, 5 g/L). For anaerobic samples that are incubated at 7 °C, plates were stored inside a BBL GasPak 100™ Anaerobe vented system (Becton Dickinson, Sparks, MD). For anaerobic samples that are incubated at 22 °C, plates were

incubated in a vinyl anaerobic chamber (Type C, Coy Laboratory Products Inc., Grass Lake, Michigan). The liquid media were overgassed with N_2, inoculated from the melted ice (~1:200 dilution), and incubated at 7 °C and 22 °C respectively. All experiments are conducted in triplicate. Cell counts reported are based on solid agar cultures after ~6 months of incubation at room temperature.

Identification of anaerobic spore formers

Each isolated was grown in MM2 liquid medium until the OD reached ~0.5. Cells were harvested and DNA was extract using a protocol for isolation of genomic DNA from gram positive bacteria in QIAGEN's DNeasy Tissue Kit (QIAGEN, Inc., Valencia, CA, USA). DNA amplifications were performed in a reaction containing ~50 ng of pure genomic DNA, 2.5 U of Taq DNA polymerase (Applied Biosystems), 5 μL of 10× PCR buffer, 2.5 mM $MgCl_2$, 200 μM each deoxynucleotide triphosphate, 1 μM each 8-forward and 1492-reverse primers, and sterile water to a total volume of 50 μL. Reaction mixtures were irradiated with ultraviolet light for 5 minutes at 254 nm (1000 J/m^2) before the addition of genomic DNA and Taq DNA polymerase to eliminate potential contamination. All negative controls with water instead of DNA template were tested. The PCR product was purified with QIAquick PCR Purification Kit (QIAGEN, Inc., CA). DNA sequencing was performed by the routine automated methods at Genewiz, Inc. (South Plainfield, NJ).

Sequencing primers, 8-forward, 1492-reverse and one internal primers, 338-forward, were used to sequence the PCR products. Sequence reads were assembled and edited by using Invitrogen Vector NTI ContigExpress. Web-based similarity searches were

performed at GenBank and the Ribosomal Database Project (RDP) [27]. Sequences were phylogenetically analyzed in an ARB database (http://www.arb-home.de/, [28]) Sequences were aligned using the Fast Aligner in ARB and the tree was constructed using the Neighbor Joining distance method with a Jukes and Cantor correction.

Atacama Desert soil sampling

All utensils were either packaged sterile or pre-treated at 240 °C overnight. The sampling sites were approached downwind, and gloves and masks worn to reduce the chance of contamination. Care was taken to minimize contamination of the experimental sites by reducing foot traffic to designated paths and areas. Soil samples were collected by spoons and scoops aseptically into sterile Whirl-Paks with the collector wearing masks, apron, and sterile gloves collected when facing the wind. The rhizosphere domain is defined as extending approximately 5 cm from the root surface in all directions. Some root nodules were collected together with soils. Sieving, homogenizing and sample handling took place in a station in Yungay. Soil samples were sealed in amber jars to protect from sunlight and moisture, transported in coolers with ice packs/dry ice or placed in dry shipper dewars for transport back for laboratory analysis.

Measure of soil water activity, pH, EH, eC and temperature in field

Most of the instrumentation and procedure were outlined in Connon *et al.* [23]. Briefly, water activity was measured by a field compatible water activity meter (Decagon, Pullman, WA). Soil hydrogen ion activity (pH), redox potential (E_H), and conductivity

(eC) were performed in the field on a 1:5 solids:deionized water suspension. An IQ150 Handheld pH/mV/temperature meter (I.Q. Scientific Instruments) or similar was used to measure pH and redox potential. Soil conductivity was measured in triplicate with an Orion 4-cell conductivity probe model 125A+ (Thermo Electron, Beverly, MA) or similar and calibrated using two traceable conductivity calibration solutions, 1413 μS/cm and 12.9 mS/cm (Cole Palmer, Vernon Hills, IL). Soil samples were analyzed for anion composition by anion exchange chromatograph (AEC) and total organic carbon (TOC). AEC was performed following the protocols of the EPA's Method 300.0 and TOC followed a modified version of a Lloyd Kahn Method by Datachem Labs (Salt Lake City, UT). A field compatible Pawkit water activity meter (Decagon, Pullman, WA) was calibrated using standards of 6.0 M NaCl (a_w = 0.76) and 13.41 M LiCl (a_w = 0.25). Following calibration, a thin layer of homogenized soil was placed along the bottom of a disposable sample cup and placed in the instrument for measurement.

Cell extraction from soils and Micro-EVA measurement

Thirty mL of autoclaved phosphate-buffered saline (PBS) (EMD Chemicals Inc., Gibbstown, NJ) with 10 mM sodium pyrophosphate and 0.1% Tween-80 (Sigma-Aldrich, St. Louis, MO) was added to 15 g of soil in a 50-mL centrifuge tube. The tube was vortexed at maximum speed for 5 minutes, with intervening 1-minute cooling in ice. The tube was then allowed to sediment for 20 – 40 minutes until a clear layer of supernatant was visible. The supernatant was aliquoted for Micro-EVA measurement. 500 μL of soil extract was filtered onto 13 mm^2 spots using a 96-well micro-sample filtration manifold (Schleicher & Schuell, Keene, NH). All these steps were carried out inside a biohazard

cabinet hood (Sterilgard III Advance, The Baker Company, Sanford, ME). Micro-EVA measurements to quantify viable *Clostridium* spores were conducted following the procedures described in the Ice Core session.

5.4 RESULTS

In this section, we will first describe the successful recovery of anaerobic spores from two depths of Greenland ice core samples, and the 16s rDNA phylogenetic analysis of these spore formers. Spectro-EVA and Micro-EVA have also been applied to quantify viable anaerobic spores from ice core samples. Due to the sensitivity of Spectro-EVA, only Micro-EVA has been applied to the soil samples from Atacama Desert.

Recovery of anaerobic spore formers from Greenland Ice Core and phylogenetic analysis

Cultivation experiments have only been conducted to older ice cores (age 10,000 years and above). Both aerobic and anaerobic cultivation were performed. For aerobic culturing, no colonies were observed in the spread plating culture on either 1/10 R2A medium, B10 medium, Butlin's medium, nitrate medium, or N-deficient medium supplemented with native ice core meltwater at different pHs 3, 5, 7, 9, 11 at 7 °C and 22 °C after 6 months of incubation. Anaerobic spread plating yielded 0 to 11 cfu/mL with both 1/10 R2A and MM2 medium at 22 °C. No colonies were found in all cultures incubated at 7 °C. Table 5.1 summarizes the anaerobic CFU counts after 6 months incubation at 22 °C.

With the liquid culture, turbidity was only observed at the MM2 media inoculated with ice core from depth 1566 m (age 10,000 years old) and depth 2517 m (age 60,000 years old), and 1/10 R2B inoculated with ice core from depth 2593 m (age 70,000 years old). Liquid from turbid MM2 cultures was replated on agar plates, and heat-shock treatment (80 °C for 15 minutes) was applied to select for spore formers. 10 different spore-forming strains were isolated from these two depths, 1566 m and 2517 m. Figure 5.2 showed the sporulating cultures of four selected strains. The vegetative cells are phase dark, rod shape, and about 4 to 6 μm long. Spores are phase bright, with about 2 μm in diameter.

Phylogenetic analysis showed that all 10 strains belong to the genus of *Clostridium*, and are closely related to each other. The closest related *Clostridium* species are: *C. putrificum* (X73442), *C. novyi* (M59100 and *C. sporogenes* (X68189), with more than 97% similarity. There are also two unknown species clustering in the group: uncultured bacterium (EU118956) and Rumen bacterium (EU124832) (Figure 5.3).

Application of Spectro-EVA on ice core samples

Figure 5.4 illustrates an excitation spectrum of a 1566 m deep ice core section. The green spectra represented the excitation spectra of 1 μM DPA with 5 μM $TbCl_3$ in water (solid line) and native ice core filtrate with 0.1 μm membrane filter (dotted line). The filtrate was shown to slightly broaden the characteristic Tb^{3+}-DPA characteristic dual-peak spectroscopic handle. Endospore spectra were compared with the dotted DPA reference. The blue, orange and pink plots represented the DPA content by autoclaving, induced

aerobic germination and induced anaerobic germination of the ice core sample, respectively. The control negative sample, shown in black, did not manifest any spectral similarity to the Tb^{3+}-DPA complex. The excitation spectrum showed that there was no DPA release from induced anaerobic germination sample, and even in the autoclaving, induced aerobic germination samples, there may be DPA release, however, the DPA concentration was too low, the dual-peak spectroscopic handle was ambiguous comparing with what we have seen from a shallower ice core sample before. The spectro-EVA analysis failed to give us conclusive results with low spore concentration samples (e.g., Greenland ice cores), due to its limit of detection.

Application of Micro-EVA on ice core samples

Micro-EVA has only been applied to quantify *Clostridium* spores from the three shallower ice cores (depth 158 m, age 600 years; depth 480 m, age 2000 years; depth 834 m, age 4000 years). Figure 5.5 showed representative Micro-EVA images of germinated *Clostridium* spores in one ice core sample and corresponding germination time course plots of two different *Clostridium* spores. DPA release during germination resulted in bright luminescent spots due to Tb^{3+}-DPA complex formation with the Tb^{3+} that was present in the surrounding agarose medium, and the intense Tb^{3+}-DPA luminescence emanating from the location of germinating endospores enabled rapid enumeration. From Micro-EVA experiments, we determined that the number of germinable *Clostridium* spores from the three depths (arranged in ascending depth order) are 1.97 ± 0.32 sp/mL, 1.21 ± 0.41 sp/mL and 1.97 ± 0.77 sp/mL (Table 5.2). From the germination timecourse (Figure 5.5 b), we observed that the spore from depth 834 m (age 4000 years old) started

to germinate within the first 5 minutes, and the luminescence intensity of the spot reached a plateau in 50 minutes. However, another spore from depth 158 m (age 600 years old) showed a different germination profile. It started with a long lag phase (~20 minutes), which was the luminescence intensity stayed unchanged, and then the intensity started to increase gradually. The rapid release of DPA started after 40 minutes, and the intensity reached a plateau after 100 minutes. The distinctive germination timecourses could distinguish different species of *Clostridium* spores.

Physical and chemical properties of Atacama soil

Hyper-arid and highly oxidizing soil in Atacama Desert, the habitat predominantly studied to date as a proxy for extraterrestrial life, differs from other extreme environments on Earth, such as permafrost, deep-sea hydrothermal vent, ice cores, in its absence of culturable bacteria and zero water activity, which precludes metabolism of all types of bacteria. The total organic carbon content varies from 0.05% to 0.1% in the soil. The pH is acidic. The redox potential (E_h) of the investigated samples varied from +260 to +480. The water activity recorded ranged from 0.01 to 0.08 during the day and peaked at 0.52 in the early predawn hours, which is also below the lowest reported a_w for microbial growth. The total organic carbon content was between 880 and 1700 μg/g soil, which were around the borderline of the limit of quantitation (1000 μg/g soil). Most desert soils contain significantly more organic carbon. There were no significant differences in water activity, soil chemistry and TOC levels between surface and subsurface samples from sites A and E. Table 5.3 summarizes the physical and chemical properties of all sampling sites.

Application of Micro-EVA on Atacama soil samples

Navarro-Gonzalez et al. has reported in the Science journal in 2003 that site A is a near-sterile region with no recoverable DNA and extremely low culturable counts [22]. We have recovered both aerobic and anaerobic microorganisms from all the sampling sites, including aerobic and anaerobic spore formers (data not shown). Here, we picked the subsurface of site E, and three depths in the mine pit for Micro-EVA study.

Figure 5.6 showed a representative Micro-EVA image of germinated *Clostridium* spores in one soil sample and corresponding germination time course plots of two different *Clostridium* spores. DPA release during germination resulted in bright luminescent spots due to Tb^{3+}-DPA complex formation with the Tb^{3+} that was present in the surrounding agarose medium. The intense Tb^{3+}-DPA luminescence emanating from the location of germinating endospores enabled rapid enumeration. From Micro-EVA experiments, we determined that the number of germinable *Clostridium* spores from all the sites tested ranged from 66 ~ 173 spores/g soil (Table 5.4). Our data suggested that the higher water activity, the higher germinable *Clostridium* spore counts. From the germination timecourse (Figure 5.6 b), we observed that the spore from depth 1 (the bottom of the mine pit) started to germinate after 5-minute lag time, and the luminescence intensity of the spot reached a plateau in 60 minutes. The germination dynamics of this *Clostridium* spore is biphasic. The spore from depth 6 (the top of the mine pit) had a much longer lag phase, ~ 30 minutes. After the lag phase, the intensity increased continuously and reached a plateau around 70 minutes. The distinctive germination time courses could distinguish different *Clostridium* spores.

5.5 DISCUSSION

We have successfully applied Micro-EVA to quantify viable *Clostridium* spores from two Mars analogs on Earth, Greenland ice cores and Atacama Desert. With the low detection limit (theoretically one germinable spore/field of view), Micro-EVA was able to identify viable spores from diluted samples, such as Greenland ice core (less than 10 spores/mL, below the limit of detection of Spectro-EVA). That explains why Spectro-EVA fails to provide distinctive signals, while Micro-EVA is able to give conclusive results. Due to the long luminescent lifetime (τ = 0.5 to ~2 ms) of Tb^{3+}-DPA complex [29], the use of time gating effectively removes background fluorescence (i.e., interferent fluorophores with nanosecond lifetimes), which eliminates potential false positive causing features and renders the image background dark. Elimination of various background signals enables a striking increase in image contrast and detection sensitivity even for the most challenging environmental extracts, such as Atacama soil samples.

The dormancy of endospores, combined with their remarkable resistance to various environmental extremes, enables them to survive for extended periods of time. Therefore endospores have been used as a model organism to understand the limit of life in various extreme environments. In microbiology, the definition of viability is often vague with the presence of microbes existing in viable-but-not-culturable, viable-but-not-germinable and germinable-but-not-viable states. A commonly accepted definition of viability is the potential of growth, however, this make it impossible to measure viability [30]. Here, we employ germinability of endospores as the indicator of viability, which will set an upper

boundary for the actually viability. The germinability of endospores is measured by the release of a unique biomarker, DPA from the spore core upon induced germination [31].

Greenland ice cores have been considered as unique repositories of microbes frozen at various points in the geologic past. Due to the extreme low temperature, Greenland ice also serves as a model to study life in another planet [3, 32]. Research on the viability of endospores in the ice core samples provides insight on the limit and longevity of life. Several efforts have been reported to recover spore formers from cold environments. For example, spore-forming bacteria have been isolated from 750,000 year old Guliya ice [33] and from a Malan Glacier ice core (0-102 m) [34, 35]. Miteva and colleagues found spore formers among the isolates acquired from GISP2 3,043 m, a visibly silty layer, which had a total microbial concentration of 1 to 9×10^7 cells/mL [14-16]. However, they note that spore formers were not dominant in their culture collection. While spore formers have been previously isolated from deep ice cores, no studies have been previously undertaken to determine the abundance of the endospores themselves in these systems. Our Micro-EVA experiments reported here successfully detect and quantify *Clostridium* spores from Greenland ice cores, which show about 1 to 2 spores/mL melt ice water. Currently, no other technologies have been able to detect viable spores at such low level, and Micro-EVA is the first technology that made such a success.

As one of the driest and oldest deserts on Earth, Atacama Desert is considered as another Mars analog on Earth [2, 22]. It serves as a proving ground for future life detection

instrumentation, and characterizing the environmental microbiology will provide an important context for data sets to be obtained by future instruments slated for flight. Furthermore, understanding which phylogenetic groups and species of microorganisms are most likely to survive and grow in Mars special regions will aid in establishing planetary protection requirements to help prevent forward contamination [36]. Bacterial spores are highly resistant to both dehydration and high temperatures and, for this reason; endospore-forming bacteria are extremely common in soils. Past effort has shown a near-sterile region (site A in the Yungay region) with no recoverable DNA and extremely low culturable cell counts [22]. However, our research group is able to recover both aerobic and anaerobic spore formers from multiple sites in Atacama Desert (data not shown). Moreover, using Micro-EVA, we are able to detect and quantify germinable *Clostridium* spores from all sample sites tested. Our data suggest that life can exist in much harsher conditions than scientists thought before, and life may exist on some special regions on another planet, such as the subsurface with less UV radiation and higher water activity.

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Table 5.1 Summary of anaerobic CFU counts in 1/10 R2A and MM2 medium after 6 months incubation at 22 ºC.

Depth (m)	Age (years)	1/10 R2A (CFU/mL ice core meltwater)	MM2 medium (CFU/mL ice core meltwater)
1566	10,000	0.6	0.3
1923	20,000	2	0
2096	30,000	0	4.1
2253	40,000	0.3	1.3
2429	50,000	0.6	0
2517	60,000	0	2
2593	70,000	11	0
2657	80,000	0	0.6
2703	90,000	0	0.6
2746	100,000	0	0
2804	110,000	1	0

Table 5.2 Summary of Micro-EVA results for Greenland Ice Core Samples.

Depth (m)	Age (years)	Time to filter (min)[a]	pH	No. of viable *Clostridium* spores per mL ice core meltwater
158	600	14	5.0	1.97 ± 0.32
480	2000	23	5.0	1.21 ± 0.41
834	4000	35	5.0	1.97 ± 0.77

a. Time to filter: the amount of time that takes to finish filtering 450 mL ice core meltwater. This parameter indicates the turbidity of ice core sample.

Table 5.3 Physical properties of all sampling sites in Atacama Desert.

Designation	Site description	Temperature, °C	pH	eH, mV	eC, µS/cm	Water activity, a_w
A	Surface	36.3	5.97	60.5	2373	0.01
	Subsurface	23.5	6.92	5.3	2740	0.06
E	Surface 2	25.1	5.89	65	2710	0.10
	Subsurface 2	19.7	6.73	15.5	2360	0.07
D1	Depth profile 1, bottom (Depth: 214 cm)	14.8	8.14	-68.2	7.12	0.37
D2	Depth profile 2 (Depth: 189 cm)	34.2	ND	ND	ND	0.20
D3	Depth profile 3 (Depth: 163 cm)	15.7	8.19	-71.8	5883	0.20
D4	Depth profile 4 (Depth: 139 cm)	23.9	ND	ND	ND	0.30
D5	Depth profile 5 (Depth: 115 cm)	20.0	8.72	-103.2	2177	0.25
D6	Depth profile 6, top (Depth: 88 cm)	20.8	8.67	-98.7	9120	0.25

Table 5.4 Summary of Micro-EVA results for Atacama soil samples.

Description	pH	Water activity (a_w)	No. of Viable *Clostridium* spores (No./g soil)
Site E subsurface	6.73	0.07	66 ± 23
Depth profile 6, Top (Depth: 88 cm)	8.67	0.25	98 ± 19
Depth profile 1, Bottom (Depth: 214 cm)	8.14	0.37	157 ± 18

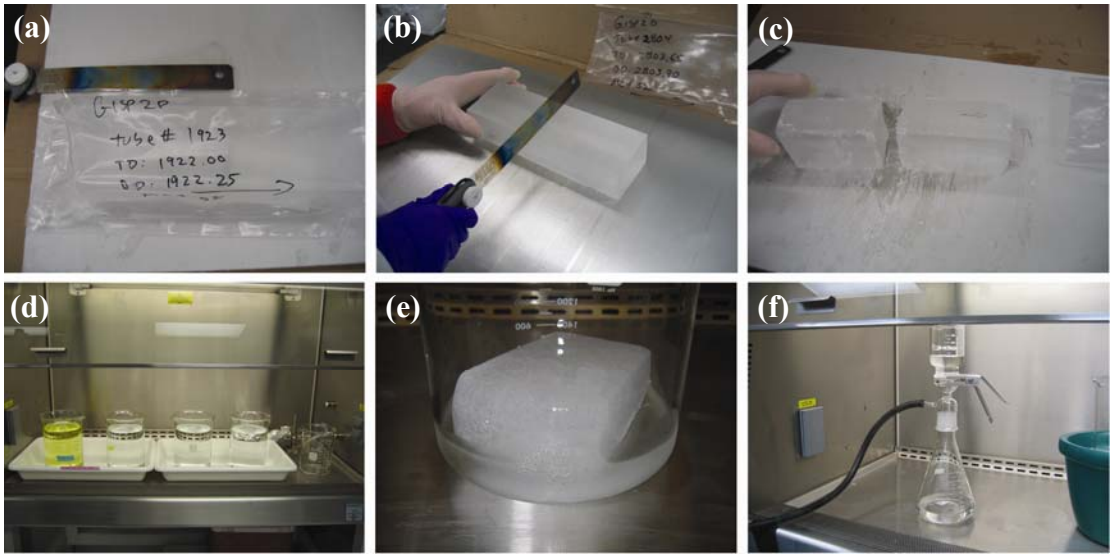

Figure 5.1 Ice core handling procedure. Ice cores were obtained form the National Ice Core Laboratory and stored in -80 °C freezer before use. **(a)** A cylinder of ice core, wrapped in sterile packaging, was placed on top of a chilled aluminum plate as a cold operating bench. **(b), (c)** A heat-sterilized saw was used to cut the core into two halves for duplicate analysis. **(d)** The cut ice core was decontaminated by a 10-s dipping in full-strength ultra-chlorox, followed by 3 sequential 10-s rinses in filter-sterilized ultrapure water. **(e)** The decontaminated ice core was allowed to melt under room temperature inside a biohazard safety cabinet. **(f)** The ice core meltwater was filtered and concentrated on 0.1-μm polycarbonate membrane filter for spectroscopic and microscopic analyses.

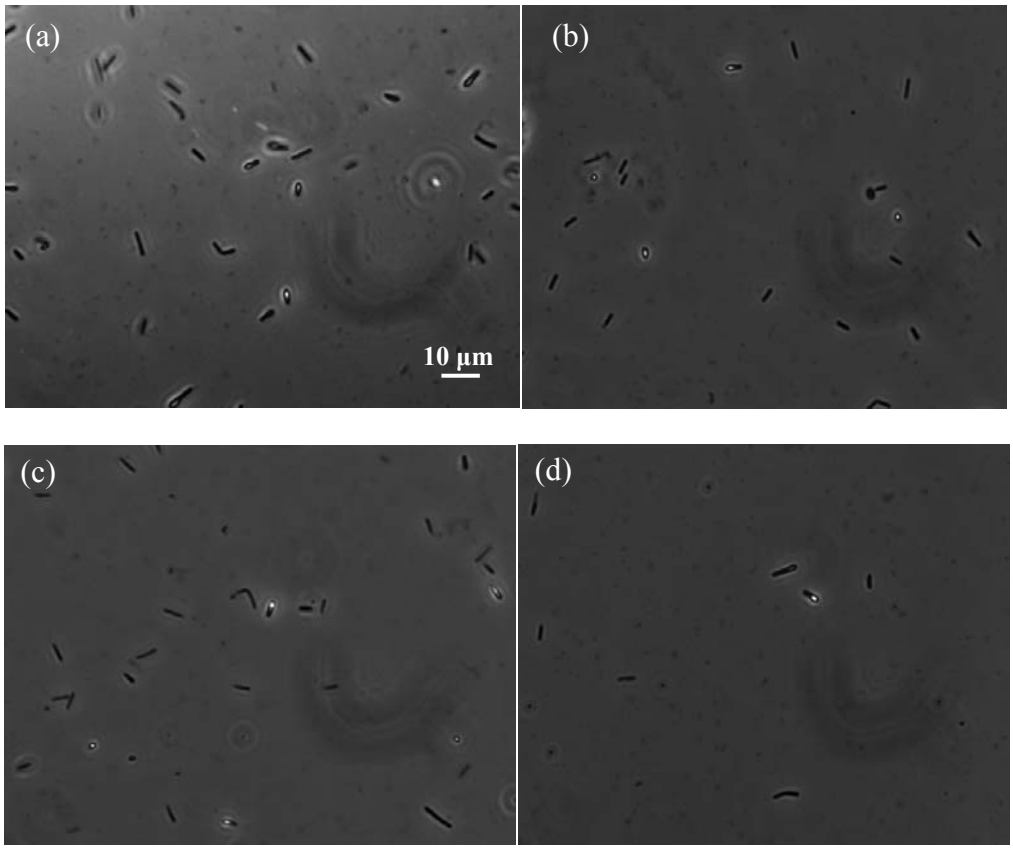

Figure 5.2 Sporulating cultures of four selected spore formers from Greenland ice core. Depth: 1566 m. Age: 10,000 years old. (a) *C. G5A-1*, (b) *C. G5A-2*, (c*) C. G5A-3*, (d) *C. G5B-1*.

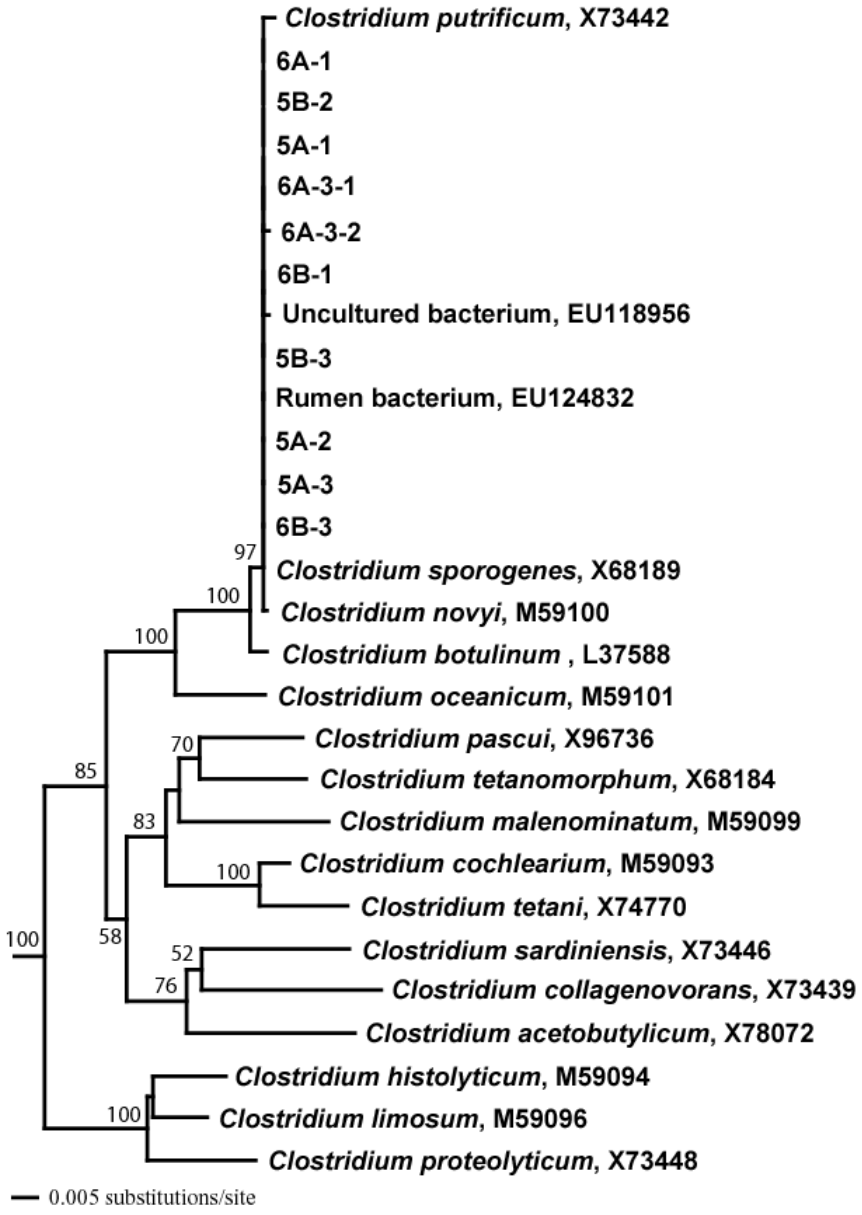

Figure 5.3 Phylogenetic tree of Greenland Ice Core isolates. 5A-1, 5A-2, 5A-3, 5B-2, and 5B-3 are isolated from depth 1566 m (10,000 years old); 6A-1, 6A-3-1, 6A-3-2, 6B-1, and 6B-3 are isolated from depth 2517 m (60,000 years old).

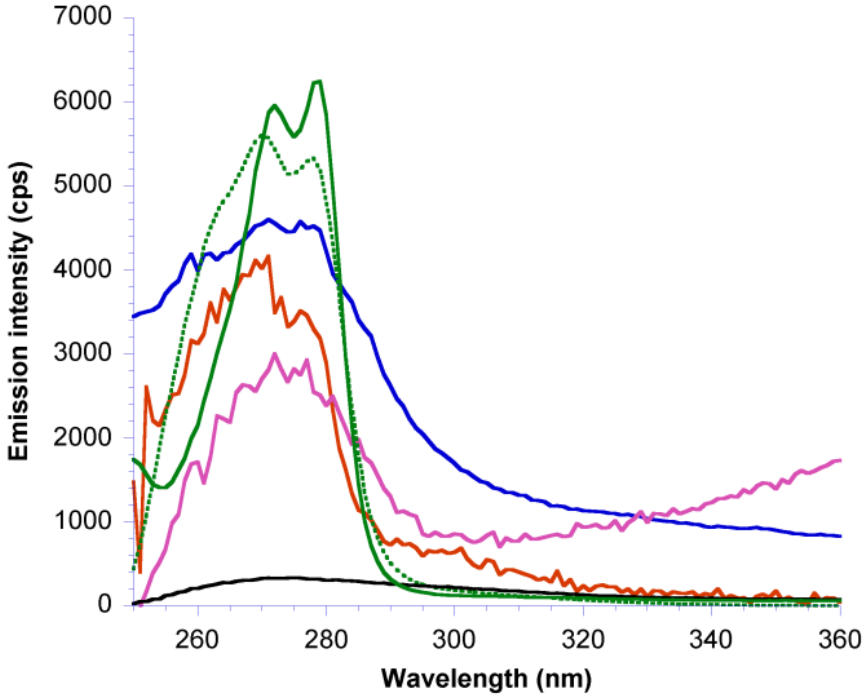

Figure 5.4 Excitation spectra of GISP2 ice core (depth 1566 m, age 10,000 years old) concentrate measured in 10 μM $TbCl_3$ following the Spectro-EVA protocol, monitoring emission intensity at 544 nm. Here are the color assignments: autoclaved sample (plot in solid blue); sample induced to aerobic germination (plot in solid orange); sample induced to anaerobic germination (plot in solid pink); 1 μM DPA (plot in solid green); 100 μM DPA in filter-sterilized ice core meltwater (plot in dotted green) and the negative control brine water control (plot in black).

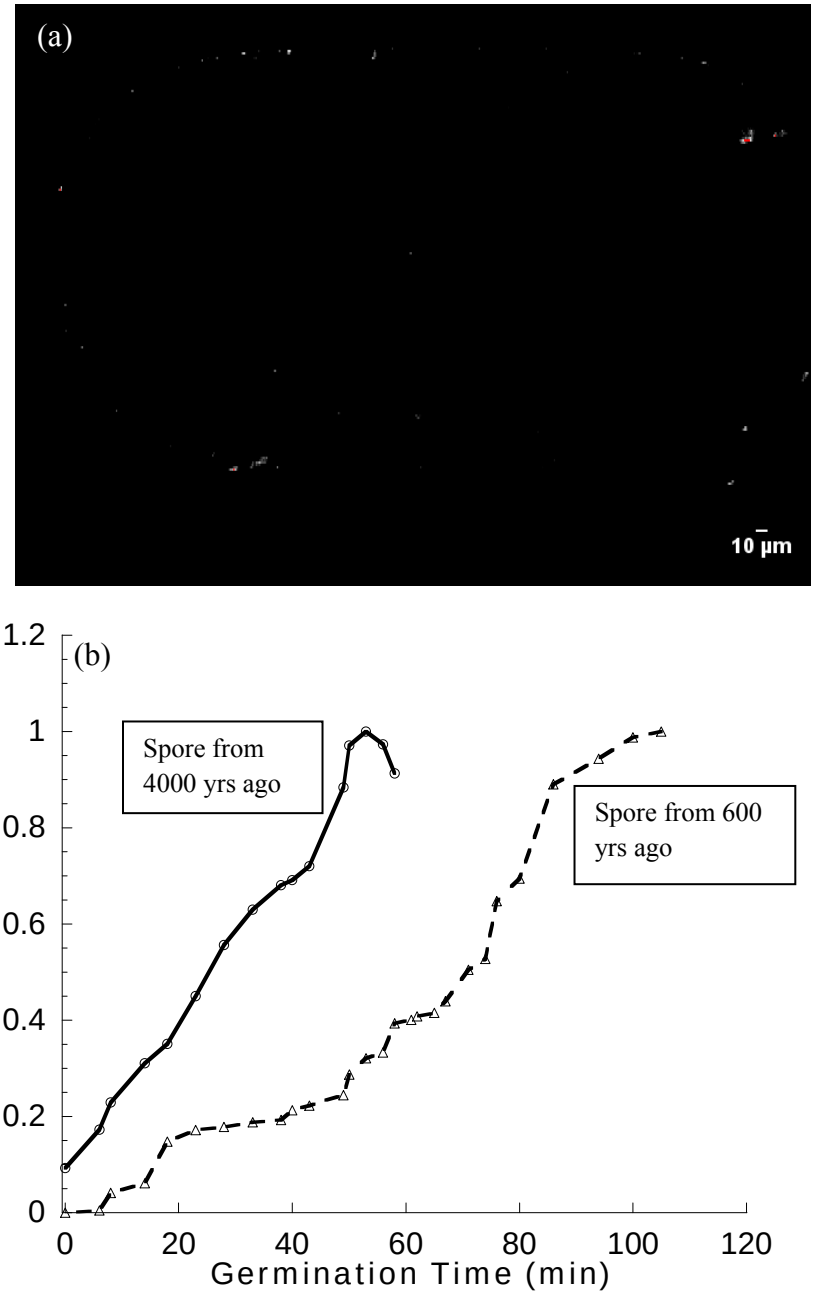

Figure 5.5 (a) Micro-EVA image showing germinated spores in the ice core sample (depth 158 m, 600 years old). Bright spots indicate germinated spores. It was taken after 120 minutes of germination. **(b)** Corresponding timecourse plots of two germinating spores.

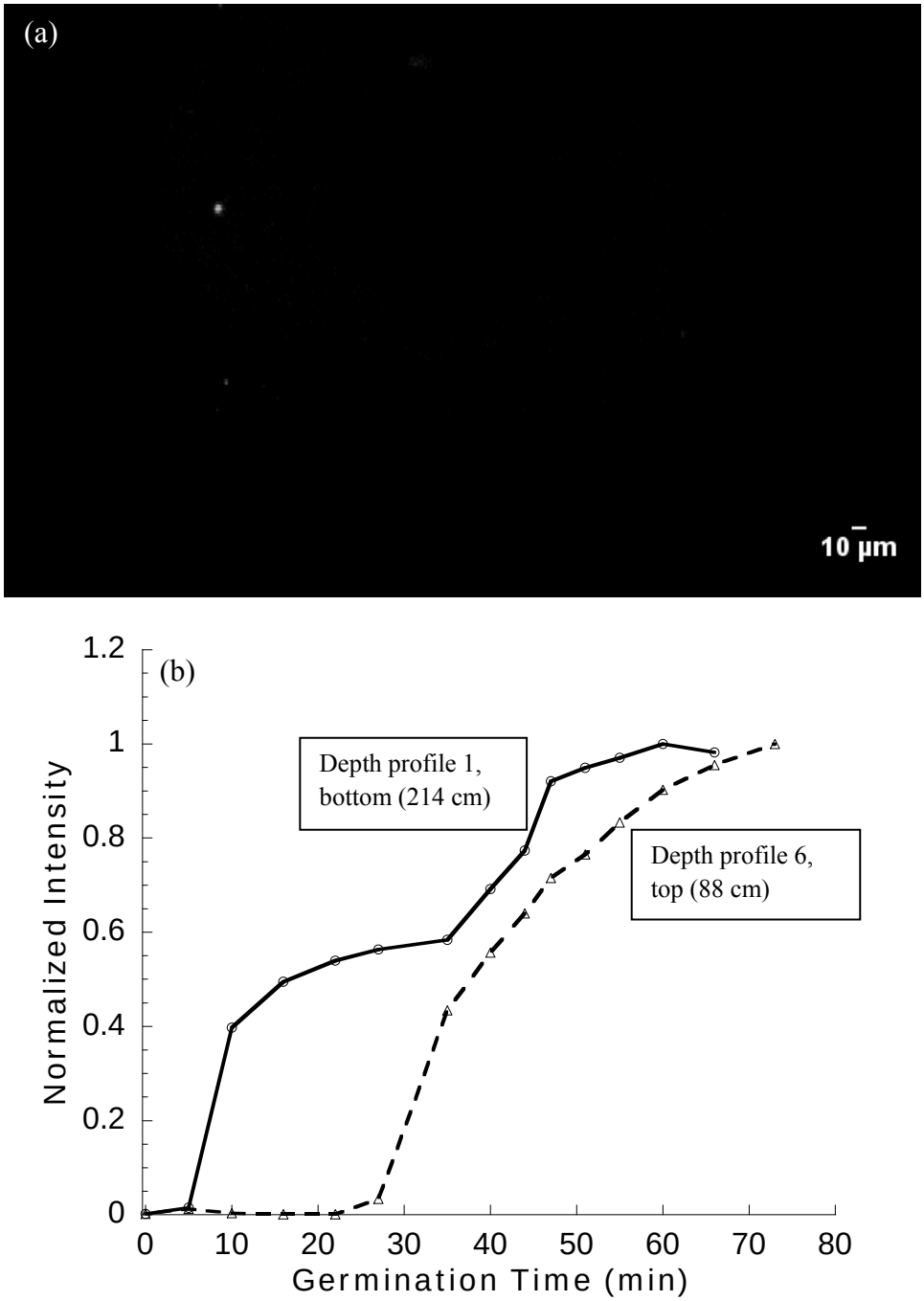

Figure 5.6 (a) Micro-EVA image showing germinated spores in Atacama soil sample (Depth profile 6, top). Bright spots indicate germinated spores, and the big bright spot represents 25 germinated spores clustering together. It was taken after 90 minutes of germination. **(b)** Corresponding time course plots of two germinating spores.

CHAPTER 6: SUMMARY

Ever since endospores had been discovered independently by Cohn, Koch and Tyndall in the latter part of the 19th century, their extreme resistance and hardy structure have fascinated the field of microbiology. They are one of the most differentiated and evolutionary capable living organisms on Earth. They are omnipresent and omnipotent, and inhabit the air, soil, deep-sea sediments, permafrost and ice cores. They can remain dormant in soil and amber for millions of years before waking up and germinating back to vegetative form. They are even postulated to have traveled billions of light years from other planets to the Earth *via* meteorites.

I focus my thesis study on the anaerobic spore former, *Clostridium*. *Clostridium* has important application in various industries. For example, *Clostridium botulinum* and *C. perfringens* are common food-poisoning agents that produce toxins which cause diseases such as botulism and human necrotic enteritis; *C. perfringens*, *C. difficile* and *C. tetani* are causative agents of gas gangrene, pseudomembranous colitis and tetanus; Some psychrotrophic clostridia are also responsible for the spoilage of chilled vacuum-packed meat; *C. acetobutylicum* has been used to produce significant amounts of acetone/butanol/ethanol by large-scale industrial fermentation; *C. perfringens* has been used as an indicator of present fecal contamination as well as a conservative tracer for recent past fecal contamination events, because it is present in large numbers in human and animal wastes.

This thesis can be broken down into four parts: (1) I developed a general protocol to produce pure *Clostridium* spores for three different *Clostridium* species. Producing pure *Clostridium* spores is a big challenge for scientists who studied this genus in the past several decades, which is also one of the reasons why the studies of *Clostridium* are not adequate. I solved this problem by optimizing the sporulation conditions for *Clostridium*, which include freeze drying the inoculums, heat-shocking the culture before incubation, and incubating at suboptimal growth temperature (3~8 ºC below optimal growth temperature). The optimized protocol yielded more than 95% degree of sporulation for three different species: *C. sporogenes* (ATCC), *C. hungatei* (ATCC), and *C. G5A-1* (isolated from Greenland ice core). Pure spore suspensions of these species were produced, and DPA was detected in all three types of spores. (2) I applied and validated a spectroscopy based endospore viability assay (Spectro-EVA) to detect viable *Clostridium* spores in liquid suspension. Spectro-EVA is based on the detection of a unique biomarker of endospores, dipicolinic acid (DPA), which is released during induced germination *via* terbium ion (Tb^{3+})-DPA luminescence. Spectro-EVA can only be applied to clean liquid samples, and its detection limit is 1000 spores/mL. However, Spectro-EVA serves as the basis for the development of a more advanced spore detection assay, Micro-EVA. (3) Micro-EVA is a microscopy based endospore viability assay, which can detect single spore germination. I validated Micro-EVA against the traditional CFU measurement to assess *Clostridium* spore viability. Micro-EVA takes the advantage of the long luminescent lifetime (τ = 0.5 to ~2 ms) of Tb^{3+}-DPA, and enables the use of time gating to effectively remove background fluorescence (i.e., interferent fluorophores with nanosecond lifetimes). Elimination of this background enables a striking increase in

image contrast and detection sensitivity even for the most challenging environmental extracts, such as soil samples. With Micro-EVA, I was also able to distinguish *Clostridium* spores from *Bacillus* spores, by using a germinant (D-alanine) that only germinates *Clostridium* spores. (4) Micro-EVA was applied to detect viable *Clostridium* spore from two Mars analogs, Greenland ice core and Atacama Desert. Our results show that Greenland ice core samples contain very low viable spore counts, ~1 or 2 viable *Clostridium* spores/mL ice core meltwater. In Atacama Desert, one of the driest deserts on Earth that has also been reported to contain near sterile soil, we found that there are around 66~157 viable *Clostridium* spores/g soil. Our preliminary conclusion is that in the extreme environments, the higher water activity, the more *Clostridium* spores are germinable (or viable). Studying the survival and longevity of *Clostridium* spores in these extreme habitats on Earth gleans invaluable information on the search for extinct or extant life on the dry and icy biosphere, Mars.

In the future, we will expand the application of Micro-EVA. Due to their ubiquitousness, *Clostridium* spores have applications in various industries: food processing, wastewater treatment, health care and pharmaceuticals. *Clostridium* spores can serve as indicators to evaluate the efficiency of a sterilization process. Micro-EVA can also be applied directly to quantify the number of viable *Clostridium* spores in real food and water samples, and to assess the viability of *Clostridium* spores in medical devices and health care facilities. Ultimately, we envision that Micro-EVA will be integrated with automated sample handling and analysis, and will enable online monitoring of various sterilization processes and facilities in the various industries mentioned above. As the application in

astrobiology, Micro-EVA will be applied to assess the sterility of spacecrafts before launching to prevent forward contamination, and be continuously used to analyze samples from more extreme environments, such as Kilimanjaro, deep sea sediments, and ultimately samples return from other planets.