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Surface Characteristics of Bacillus Spores

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Surface Characteristics of Bacillus Spores

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Abbreviations

A	Appendages
BC	*Bacillus cereus*
BHI	Brain Heart Infusion broth
BL	*Bacillus licheniformis*
BS	*Bacillus subtilis*
cps	Counts per second
DPA	Diplicolinic acid
EEM	Excitation-Emission Matrices
Em	Emission
Ex	Excitation
FL	Fluorescence Intensity
nm	Nanometers
pk	peak
SEM	Scanning Electron Microscopy/ Scanning Electron Micrograph
TLS	Total Luminescence Spectroscopy
TSA	Tryptic Soy Agar
VP	*Virgibacillus pantothenicus*

Abstract

SURFACE CHARACTERISTICS OF BACILLUS SPORES

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

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Rapid isolation and identification of spores from various environmental samples is necessitated because anthrax spores can be used as biological weapons. The hydrophobic nature of spores may allow for their rapid concentration and partial purification from contaminating materials. In this study, spores from four taxonomic groups of *Bacillaceae* were isolated, purified and characterized for hydrophobicity by hexadecane partitioning, surface morphology by scanning electron microscopy, and steady-state fluorescence by spectroscopy. The morphology of spores was similar within taxonomic groups and dissimilar between groups. Spore hydrophobicity ranged from 0.3% to 65% and all spores had fluorescence emission peaks at 335 nm and 450 nm. The excitation maxima for the peak at 450 nm were shifted to higher wavelengths for the least hydrophobic spores. Regression analysis demonstrated a correlation between the taxonomic identity, as established by fatty acid analyses, and hydrophobicity. Hydrophobicity can be used to help isolate spores from complex environmental samples and intrinsic fluorescence is helpful in discriminating the taxonomic groups.

INTRODUCTION

Members of several bacterial genera, including those of *Bacillus* and *Clostridium,* have a unique survival mechanism when environmental conditions are inhospitable (Moat, et al., 1995, Sneath, 1986). These species form endospores, commonly called bacterial spores; these spores are the most resilient life form. Spores keep the bacteria in a state of dormancy until optimal environmental conditions allow for survival and growth of the vegetative bacteria (Driks, 2002, Wipat, et al., 1999).

Cano et al. (1995) extracted and germinated an ancient relative of *Bacillus sphaericus* from an extinct bee, encased in a piece of Dominican amber which was approximately 25 to 40 million years old. Vreeland et al. (2000) further illustrated the survival of spores by recovering and germinating a halotolerant *Bacillus* sp. from a 250 million year old salt crystal. DNA analysis determined the lineage of this specimen to be related to *B. marismortui* and *Virgibacillus pantotheniticus*.

One spore is formed from one vegetative bacterium. Each spore is composed of a protoplast, a protoplast membrane, cortex and three coat layers (Turnbill, 1996). This multilayered outer shell excludes macromolecules. An additional heat and chemical resistant component in the cortex, peptidoglycan, increases the spore's resiliency (Turnbill, 1996). *Bacillus* spores central protoplast

contains diplicolinic acid, (DPA), the component necessary for high temperature tolerance (Turnbill, 1996). Slieman et al. (2001) found that approximately 10 percent of a spore's dry weight is DPA. DPA exists as a calcium complex. Rosen et al. (1997) developed a terbium chloride assay to identify and quantify endospore concentrations utilizing this DPA-Ca^{++} complex. The reaction between the calcium diplicolinic acid complex and terbium chloride results in a terbium (III) anion. This terbium anion is photoluminescent when in the presence of the DPA-Ca^{++} complex and is easily recognized.

Several species of *Bacillus* and *Clostridium* are important to human and animal health. Some examples include *C. botulinium, C. tetanus and C. perfringenes* which are the causative agents for botulism, tetanus and gas gangrene, respectively. *B. anthracis*, the agent of anthrax, is a zoonotic disease which primarily affects grazing animals and can also be a dangerous pathogen to humans. *B. anthracis* spores are known to survive along livestock trails in the United States causing frequent outbreaks in states from Texas to South Dakota (CDC, 2001). Spores of *B. anthracis* are excellent potential biological weapons. Two recent events illustrate this in which aerosolized anthrax laced letters were delivered to Capitol Hill and to an office building in Florida (Weis, et al., 2002; CDC 2002; CDC, 2001). Members of the *B. cereus* group can cause food poisoning in humans and related forms (eg. *B. thuringenesis*) are used in biological control of insect pests.

As mentioned above, the protoplast is enveloped by the cortex, followed by three protein coats (Turnbill, 1996). However, members of the *B. cereus* group, which includes, *B. anthracis*, have an additional protective layer, the outer-most exosporium. These multilayered outer structures, which can make up half of the spore's weight, provide protection for the spores from chemical, physical and enzymatic degradation (Turnbill, 1996).

There are several reliable assays used to characterize and identify spores. Genetic identification relies on PCR- DNA sequencing to identify the species by their nucleotide sequences (Hansen, et al. 2001; Kolbert, 1999). Phenotypic identification relies on physiological profiling such as the Sherlock Microbial Identification System (MIDI Inc., Newark, DE), and BIOLOG, (Biolog, Inc., Hayward, CA). The MIDI system utilizes fatty acid analysis of the bacterium, and assigns a numerical value to the results, called a similarity index. This similarity index is then compared to an internal library which chooses the most similar identities for the microbes in question. BIOLOG is a cell-based test for utilization of carbon sources. It requires a 96-well plate with multiple carbon sources from which color changes are compared to a Biolog library to determine the identification of the bacterium. Steady-state fluorescence is a powerful tool for distinguishing differences in molecules and macromolecules and the technique may prove useful in examining spores. Fluorescence spectroscopy utilizes emission peaks to characterize spores. It is a sensitive technique because excitation is performed at a single wavelength. The resulting emission data are recorded at a

longer wavelength. Bronk and Reinisch (1993) concluded that initial microbial identification could be generated using fluorescence spectroscopy. All spore assays are influenced by environmental contaminants (Kuske, et al., 1998, Balser, et al. 2002, Gamo et al. 1999) and each would benefit from isolation, concentration and purification.

One technique that may aid in spore isolation and concentration from environmental samples is to use partitioning into hexadecane or some similar hydrophobic material whereby hydrophobic spores would partition into the hydrocarbon and hydrophilic spores would remain in the aqueous phase. Rosenberg et al. (1980) found pure laboratory bacterial cultures exhibited different degrees of hydrophobicity depending on genus and species nearly a quarter century ago. Koshikawa et al. (1989), using hexadecane titration, found that clean laboratory isolates exhibited different degrees of hydrophobicity.

Because *Bacillus* spores have been used as biological weapons, any information on their isolation, recovery and growth would be valuable. Techniques to enhance recovery and detection in the environment would be a valuable tool. The objectives of this project were to develop a better understanding of surface characteristics of bacterial spores and to determine if these differences could be used to enhance the isolation, detection and identification of spores. Spores from laboratory stocks and new environmental isolates were compared using light and electron microscopy, total luminescent spectroscopy (TLS) and relative surface hydrophobicity and hexadecane partitioning.

MATERIALS AND METHODS

Bacillus isolation and identification

Soil samples from three locations were used for selection of fresh field isolates to obtain organisms that had not been subjected to extensive laboratory cultivation or selection. These sites were Richmond, Virginia; San Antonio, Texas and Albuquerque, New Mexico. Soil samples were suspended in Brain Heart Infusion broth (BHI), heat shocked for 15 minutes at 80° C to kill all vegetative forms and stimulate spore germination. Grown for 8 hours under aerobic conditions at 37° C with gentle shaking, the mixed cultures were subjected to streak plate isolation on Tryptic Soy Agar (TSA). Individual colonies were purified further and/or selected by two additional streak plate cultures on TSA. One soil sample from Richmond was maintained as a primary mixed culture after heat-shocking. This sample, designated as "mix", was used directly for spore production without additional selection, culture or isolation. Identification of isolates by MIDI fatty acid analysis was performed using the Sherlock Microbial Identification system (MIDI, Inc., Newark, DE). Final identification was made based upon comparison of the fatty acid profile of the isolate with profiles in the MIDI library. Identities of the 14 isolates are shown in Table 1. Nine were new isolates from soil, four were laboratory cultures and one was a mixture from soil.

Table1. *Bacillus* Isolate Identification

Code	Isolate	Location	Culture Source
BC1	*Bacillus cereus (GC Group A)*	San Antonio, TX	soil
BC2	*Bacillus cereus (GC Group A)*	Richmond, VA	soil
BC3	*Bacillus cereus (GC Group A)*	Albuquerque, NM	soil
BL1	*Bacillus licheniformis*	San Antonio, TX	soil
BL2	*Bacillus licheniformis*	Richmond, VA	soil
BS1	*Bacillus subtilis*	Albuquerque, NM	Soil
BS2	*Bacillus subtilis*	Pine Bluff Arsenal	Lab
BS3	*Bacillus subtilis* (PY-79)	Harvard University	Lab
BS4	*Bacillus subtilis* (CVO-1000)	Harvard University	Lab
BS5	*Bacillus atrophaeus (B. globigii)*	Pine Bluff Arsenal	Lab
MIX	*Soil Mixture*	Richmond, VA	Soil
VP1	*Virgibacillus pantothenicus*	Albuquerque, NM	Soil
VP2	*Virgibacillus pantothenicus*	San Antonio, TX	Soil
VP3	*Virgibacillus pantothenicus*	Richmond, VA	Soil

Included in the 13 purified isolates were three *B. cereus* of the GC Group A from soil, two *B. licheniformis* from soil and three *Virgibacillus pantothenicus* from soil. The other five were members of the *B. subtilis* group.

In recent years the *Bacillacea* family has undergone some taxonomic revisions. In 1998, *Bacillus pantothenticus* underwent genotypic and phenotypic testing by Heyndrickx et al.(1998) and based on their results, it was reclassified into a separate new genus, *Virgibacillus.* This change warranted a phylogenetic investigation of the family. Bergey's Manual identifies the hierarchy for the bacteria as: Domain: Bacteria, Phylum BXIII- *Firmicutes*, Class III *Bacilli*, Order I *Bacillales*, Family I *Bacillaceae*, Genus I, *Bacillus*, with *Virgibacillus* now listed as Genus VIII (Boone, et al., 2001). Bergey's Manual now recognizes *Virgibacillus* as its own genus; however, it is still a member of the *Bacillaceae* family (Boone, et al., 2001). The *B. subtilis* isolate coded BS 5 was identified as *B. atrophaeus*, previously known as *B. globigii*.

Spore production and preparation

Bacillus spores were germinated in BHI. Cultures were spread on sporulation agar plates and allowed to grow for one week at 37°C under aerobic conditions. They were harvested by scraping and suspended in sterile DI water. The suspensions were subjected to lysozymal degradation at 37°C for 2 to12 hours, and then the suspensions were subjected to differential centrifugation three times at 9,000g for 15 minutes, and suspended in sterile DI water.

A negative stain was performed to assess the purity of the spores. If vegetative cells were still present, the lysozyme and centrifugation processes were repeated until the spore suspensions were free of vegetative cells (Riesenman, et al. 2000).

Light and electron microscopy

Samples of wet spores and bacterial cultures were examined by light microscopy using Gram stain, malachite green endospore stain and nigrosin for negative staining. Scanning electron micrographs (SEM) were taken of each final spore preparation that was used for fluorescence spectroscopy and hydrophobicity measurements to validate purity based on homogeneity of the spores and to determine surface morphology. Lyophilized spores were attached to double sided tape that was adhered to a metal stub then sputter-coated with gold to make the spores conductive. Spores were placed in a vacuum chamber and examined with a JEOL JSM-820 scanning electron microscope. Representative photomicrographs were taken of each spore sample at a magnification of either 7,000X or 10,000X.

Fluorescence Spectroscopy

Total Luminescent Spectroscopy

Spore preparations were subjected to analysis by total luminescent spectroscopy (TLS) to determine if hydrophobicity or spore morphology was correlated with spectral photoluminescence. TLS simultaneously measures excitation and emission (wavelengths in nm) and intensity spectra (counts/sec.). These measurements are then plotted three-dimensionally in the form of excitation-emission matrices (EEM). Steady-state fluorescence intensities emitted by spores were analyzed using a FluoroLog-3 spectrofluorometer (JY Horiba). Contour and surface plots of these intrinsic spore signatures were constructed using Matrix Laboratory (Mat-Lab) computer software. The instrumental parameters used for the excitation-emission matrices (EEMs) included the excitation range of 280 to 450nm at 10nm increments, and the emission range of 300 to 575nm also in 10nm increments. The three-dimensional representations were plotted with the x-axis as excitation, y-axis as emission and the z-axis as fluorescence (FL) intensity. TLS data established the optimum fluorescence peaks of Ex 290/ Em 335nm and one at Em 450 (Ex variable). Once these optimum wavelengths were determined, single wavelength scans were performed to allow direct comparison of intensities for the spore preparations.

Single Excitation Fluorescence Spectroscopy

An optimal excitation wavelength was determined by utilizing the TLS data. Single emission scans were used for the Em 335 peak using detection at Ex 290 nm with emission wavelengths from 300 – 450 nm at 1nm intervals. These two-dimensional data files were then graphed using Microsoft Excel spreadsheets, where the x-axis represented the emission wavelengths and the y-axis was FL intensities (cps).

Single Emission Fluorescence Spectroscopy

Similarly, an optimal emission wavelength was chosen based on the TLS data. The emission wavelength used for each sample was 450nm. Single excitation scans were used for the Em 450 peak using excitation wavelengths from 280 – 400 nm at 1nm intervals. These two-dimensional data files were then graphed using Microsoft Excel spreadsheets, where the x-axis represented the excitation wavelengths and the y-axis was FL intensities (cps).

Partition Titration with Hexadecane

The hexadecane partitioning technique, developed by Rosenberg et al. (1980) and used by Koshikawa et al. (1989) was performed on spore suspensions during this research. The spore samples were diluted in sterile DI water to a concentration of 0.5 OD_{610}. Once diluted, 3ml of each suspension was placed in 5ml Falcon (Fisher Scientific, Pittsburg, PA) tubes.

Then hexadecane (Sigma-Aldrich, St. Louis, MO) in the following amounts was added to the tubes, 0.1ml, 0.2 ml, 0.3ml, 0.4ml, 0.5ml, 0.75ml, 1.0ml, and vortexed for one minute until mixed. After 15 minutes, which allowed the hexadecane and water to separate, the aqueous phase was removed and the absorbance of the aqueous phase was measured at 610 nm. The percent change in absorbance was calculated to determine the hydrophobicity of each spore suspension.

RESULTS

Scanning Electron Microscopy

Comparison between taxonomic groups revealed profound differences among the spore isolates. Representative SEM photomicrographs of each morphological type are shown in Figure 1. Visual examination revealed the surface structure of all spores within a group was different from the structures observed for spores of the other taxonomic groups. SEM provided support that each spore suspension was taxonomically pure and contained only spores. Spores of the three *B. cereus* isolates had a relatively smooth surface with an exosporium. The exosporium prevented the clear delineation of one spore from the next as was readily apparent for the other three groups. The surface of the two *B. licheniformis* isolates was covered with appendages which are characteristic of the species. The five *B. subtilis* isolates all had uniformly smooth spore coats with gently flowing ridges and did not exhibit an exosporium or appendages. Spores of the three *Virgibacillus pantothenicus* isolates were approximately two times larger than the *Bacillus* sp. spores and their surface was smooth with four prominent ridges traversing their lateral, longitudinal surfaces. The “mix” sample contained spores of various sizes and morphologies as would be expected for a primary mix culture from soil.

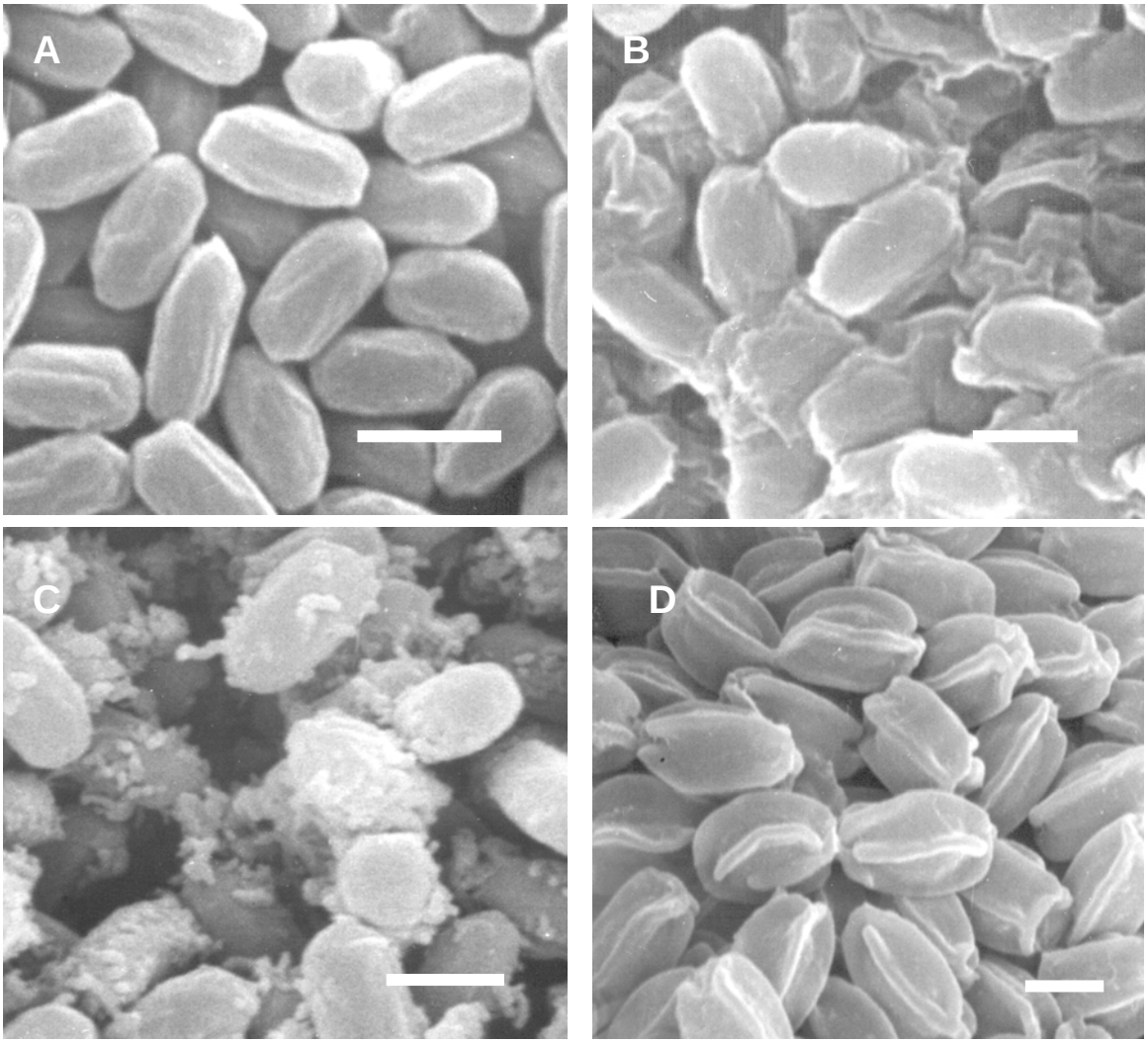

FIG. 1. Scanning electron micrographs of purified spores showing the surface texture of representative types. (A) *Bacillus subtilis* (along with *B. atrophaeus /B. globegii)* spores were uniformly smooth with gentle flowing ridges and showed no evidence of an exosporium, (B) *B. cereus* spores were uniformly smooth with a loosely fitting exosporium, (C) *B. licheniformis* spores were covered with appendages, and (D) *Virgibacillus pantothenicus* spores were smooth with four prominent lateral ridges traversing their longitudinal surfaces. The bar represents 1μm.

Partition titration with Hexadecane

Partition titration with hexadecane was performed on each aqueous spore suspension to determine the relative hydrophobicity of each isolate. This was done as another tool to characterize each spore isolate. Results are shown in Figure 2 where the "mix" is compared with the five *B. subtilis* isolates (Fig. 2, panel A) and with all other isolates (Fig.2, panel B). Because the "mix" preparation was shown by SEM to contain a mixture of spore types, this sample was used as the primary reference within this study. The "mix" had an endpoint for partitioning into hexadecane of 28%, thus any spore preparation with a hydrophobicity percent value higher than 28% was considered relatively hydrophobic and any spore preparation with a value lower than 28% was considered relatively hydrophilic.

The five members of the *B. subtilis* group showed the greatest diversity of any group, with spore hydrophobicity ranging from 0.3% to 65%. Spores of two isolates, BS2 and BS4, were relatively hydrophilic; one, BS3 (23%), was similar to the "mix" and two, BS1 and BS5, were quite hydrophobic. Spores of all three *Virgibacillus* isolates were relatively hydrophilic, 6% to 13%. Two spore preparations of *B. cereus*, BC1 and BC2, were hydrophilic with values of 15% and 10%, respectively. The other, BC3, was similar to the mix (27%).

Two of the spore samples were further titrated beyond 1ml of hexadecane up to 3.0 ml, which represented a final ratio of 1:1, water to hexadecane. This was done to confirm that either the slope of the line and/or the endpoint did not change with addition of more hexadecane. BS2 and BS5 were chosen since they represented the second least (BS2, 6%) and the second most (BS5, 49%) hydrophobic of all spores. The slope of the titration lines for BS2 and BS5 did not change and their endpoints remained the same.

Spore hydrophobicity was compared with taxonomic identity using similarity indices from MIDI fatty acid analysis. This comparison is shown in Figure 3. Regression analysis revealed a strong correlation between taxonomy and hydrophobicity.

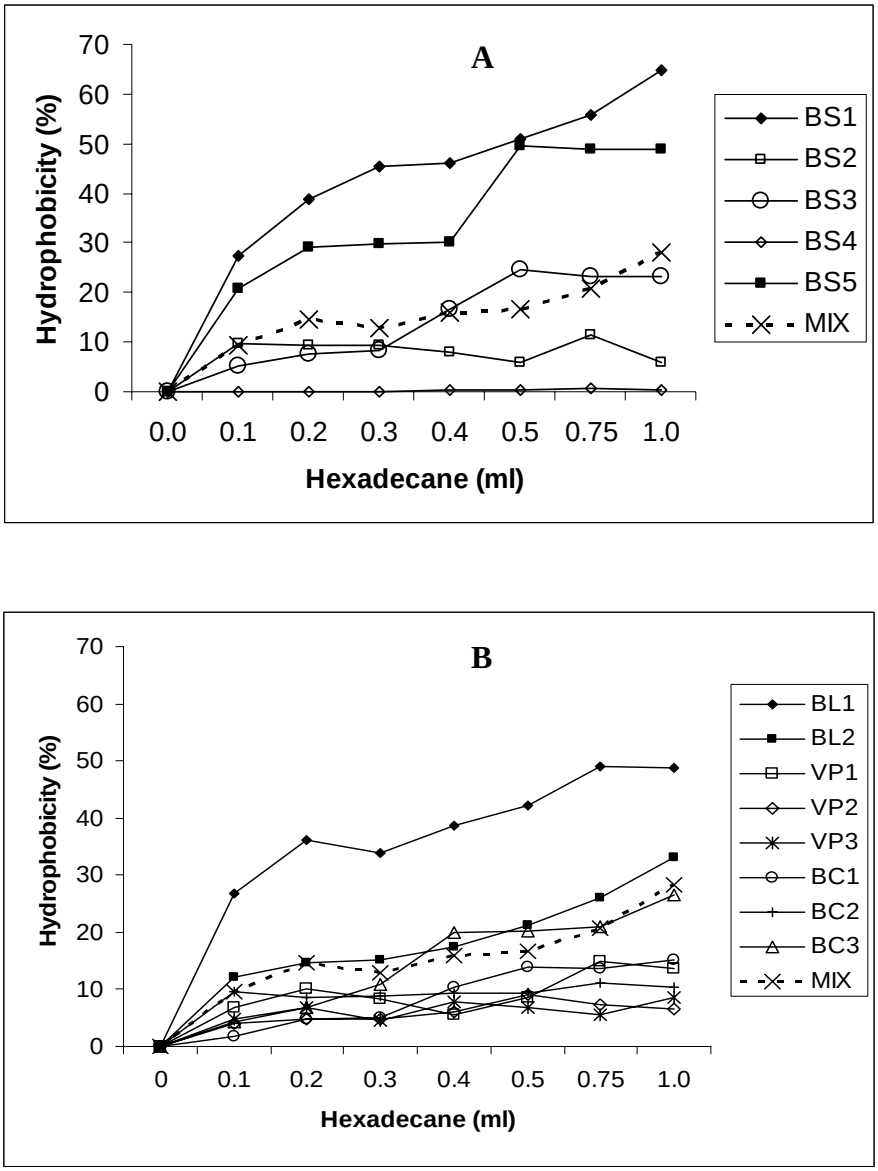

FIG. 2. Hexadecane Titration of Spores. The top panel (A) includes all *B. subtilis* isolates compared with the MIX. The percent hydrophobicity was the decrease in percent of the aqueous phase at A_{610} which was the result of spores partitioning into the hexadecane. Spores with values above the MIX (see dashed line) were classified as relatively hydrophobic and those with values below were classified as relatively hydrophilic. Shown in the bottom panel (B) are all other spores used.

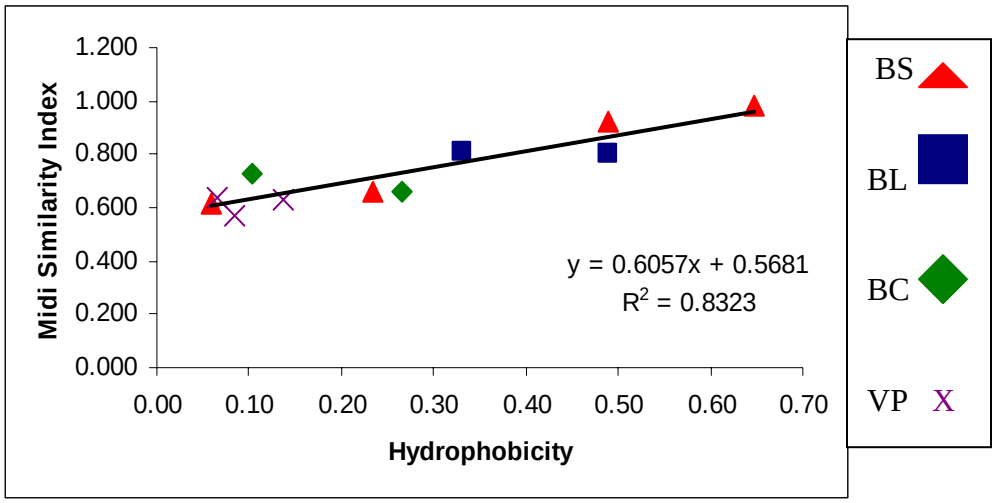

Figure 3. Comparison of MIDI similarity indices to percent hydrophobicity of spore isolates. Regression analysis indicated a strong relationship between taxonomic groups and hydrophobic characteristics.

Fluorescence Spectroscopy

Representative TLS data are shown in Figure 4 as contour plots and surface plots. Spores from all groups, including the "mix", had two peaks of fluorescence emission. The centroid of one peak was at Ex 290 nm and Em 335 nm and the other was a broad peak at Em 450 nm. All spores shared the same optimum excitation wavelength for the peak of emission at Em 335 nm. However, the excitation optima for the peak at Em 450 nm exhibited a broader range from Ex 320-369 nm.

These data are presented in Table 2 along with hydrophobicity data and surface characteristics. The excitation maxima for the Em 450 nm peak ranged from Ex 320 to 369 nm for the five isolates of *B. subtilis*. Similar variation was detected for the other two *Bacillus* groups (*B. cereus,* Ex 322 to 363 nm; *B. lichenformis,* Ex 320 to 352 nm).

The Ex290/Em 335 peaks had intensity values for the samples ranging from 0.8 - 6.5 x 10^6 cps. This peak is consistent with most biological specimens that contains aromatic amino acids, primarily tryptophan and tyrosine. Visual inspection showed no relationship between intensity of this peak with hydrophobicity or morphology.

The Em 450 peaks had intensity values for the samples ranging from 0.2 – 2.7 x 10^6 cps. Visual inspection of these data (see Table 2) suggest a relationship between hydrophobicity and fluorescence excitation.. The most hydrophilic spores (0.3% – 13%) showed a cluster relative to excitation at higher wavelengths (Ex 362 -369 nm).

For some isolates the Em 335 nm peak intensities were higher than the Em 450 nm peak intensities (see Fig 3, panels A and C), whereas with other spores the pattern was reversed (see Fig. 3, panels B and D). To compare the two peaks, ratios were determined using the respective intensity maximum from each peak and are shown in Table 2. Visual inspection showed a relationship among taxonomic groups of soil isolates between peak intensity ratios and hydrophobicity. For instance, all three *B. cereus* isolates had a ratio of 0.1. Isolates of *B. licheniformis* and *V. pantothenicus* were similarly low (0.1-0.5). *B subtilis*, on the other hand, again showed the greatest level of diversity, ranging from 0.3 to 2.3. The mix had a ratio of 1.4.

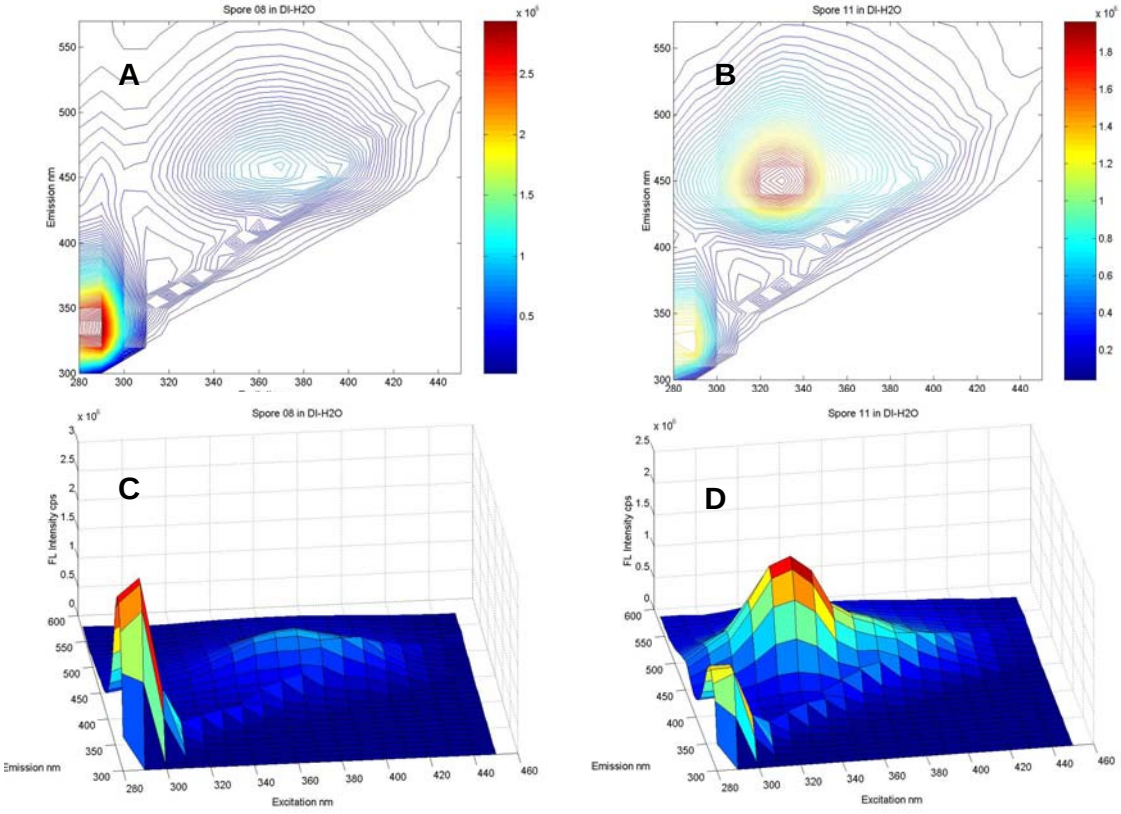

FIG. 4. Intrinsic Fluorescence of Spore Isolates. Panels A and B are contour plots of total luminescence spectra (TLS) of spores of two different *Bacillus subtilis* isolates.. Shown below each one is the corresponding surface plot (see Panels C and D).

Table 2. Hydrophobicity, Surface and Fluorescence Characteristics

ID	% Hydrophobic	Surface Code	Fluorescence Intensity 290nm Excitation pk (nm)	cps ($X10^6$)	450nm Emission pk (nm)	cps ($X10^6$)	Ratio 450/290
BS1	64.7	S	335	2.7	366	0.7	0.3
BL1	48.9	S,A	336	3.3	320	1.0	0.3
BS5	48.9	S	337	1.2	320	2.7	2.3
BL2	33.1	S,A	336	2.5	352	0.3	0.1
MIX	28.2	S,R,A,X	335	0.8	359	1.1	1.4
BC3	26.6	S,X	335	6.0	349	0.8	0.1
BS3	23.4	S	336	2.0	322	2.4	1.2
BC1	15.2	S,X	335	6.5	362	0.8	0.1
VP1	13.6	S,R	338	3.5	363	0.3	0.1
BC2	10.4	S,X	337	4.2	364	0.6	0.1
VP3	8.5	S,R	337	4.3	364	2.2	0.5
VP2	6.6	S,R	337	1.1	362	0.2	0.2
BS2	6.0	S	336	1.8	369	1.0	0.6
BS4	0.3	S	336	2.3	366	2.5	1.1

S=smooth
R=ridges
A=appendage
X=exosporium

DISCUSSION

Bacillaceae is a large, diverse family of bacteria with one characteristic in common; they all form spores. It was no surprise that the fourteen spore preparations used in this study reflected that diversity relative to (1) surface morphology, (2) hydrophobicity and (3) intrinsic fluorescence characteristics. In contrast to most previous reports that use laboratory strains, ten of the spore types used in this study were new isolates from soil that had not been subjected to years of selection and growth in the laboratory. These new field isolates showed greater homogeneity within their respective taxonomic group than did the five representatives from the *B. subtilis* Group, most of which had been grown in the laboratory for many years. It may be difficult to compare results of hydrophobicities from one study with those of another based on taxonomy alone. The results of this study suggest that variation between laboratory strains of the same species are far greater than natural variation that occurs in geographically isolated, but taxonomically similar stains existing in nature.

The results reported here confirm the observations of others that most bacterial spores are relatively hydrophobic. Doyle et al. (1984) reported that *B. cereus* cells were hydrophilic but their spores were hydrophobic. Hydrophobicity of their two *B. cereus* spores was 56% (strain T) and 63 % (strain 9634).

Others have described values for the spores of T strain as 85% (Koshikawa et al., 1989) and 95% (Wiencek, et al., 1990). *B. cereus* spores used in this project were all new isolates from soil that had lower hydrophobicity values of 10%, 15%, and 26%. The differences may be attributable to changes that occur through repeated growth and selection in the laboratory as is suggested by data reported for *B. subtilis*. Wiencek, et al. (1990) examined 7 strains of *B. subtilis* and reported values ranging from 19% to 95%. Four of the five *B. subtilis* used in this study were also laboratory strains; they also had a wide range from 0.3% to 65%. We found a similar hydrophobicity (49%) as Wiencek et al. (1990) for the one common strain used in both studies, *B. globigii* (also known as *B. atrophaeus, B. subtilis* ATCC 9372). Lysozyme treatment can influence hydrophobicity values for some spores, but not others. Doyle et al. (1984) showed that spores of *B. subtilis*, strain 168, increased from 14% to 35% after lysozyme treatment. They suggested that cell wall components, especially peptidoglycan, were still present before lysozyme treatment and contributed to lower hydrophobicity values. All spores used in the present study were treated with lysozyme to remove all cell walls.

Scanning electron microscopy (SEM) confirmed the purity of each spore isolate and the lack of cell walls or other debris. SEM showed the same surface morphology within each taxonomic group. Surface ultrastructure of the five *B. subtilis* isolates were all smooth with no appendages. Their structure, and the structures of spores of *B. licheniformis* and *B. cereus,* was consistent with descriptions and micrographs previously reported by many others. *B. licheniformis*

spores were smooth with appendages and those of *B. cereus* were smooth with an exosporium. *Virgibacillus pantothenicus* was smooth with four prominent lateral ridges. To our knowledge, these are the only scanning electron micrographs of this species.

Examination of spores with fluorescence spectroscopy showed two peaks for each isolate. One was a peak located at Ex 290 nm/Em 335 nm and the other was at Em 450 nm. Bronk and Reinisch (1993) also found two peaks of fluorescence in the same relative emission wavelength regions, Em 330 nm and Em 470 nm. They attributed the Em 330 nm peak to aromatic amino acids, primarily tryptophan, but they did not identify the source of fluorescence for the peak at Em 450 nm. Bronk and Reinisch (1993) reported similar fluorescence for spores of *B. subtilis, B. cereus, B. megaterium* and *Clostridium perfringens* using single excitation scans where the Em 330 nm peak was predominant. They suggested that total luminescence spectroscopy (TLS) should be used in the future to compare spore types. Using TLS we report here that spores were different with respect to (1) the excitation maxima for the Em 450 nm peak and (2 & 3)in the relative intensities and ratios of the Em 335 and Em 450 peaks. The most hydrophilic spores had similar excitation optima for the Em 450 peak, between 360 nm to 370 nm, suggesting some relationship existed between hydrophobicity and fluorescence. Since the nature of fluorescence for this Em 450 nm peak has not been ascribed to a particular molecule(s), the relationship between surface charge (e.g. hydrophobicity) and fluorescence remains unknown. All spores of *B. cereus* had a

higher fluorescence emission at 335 nm than at 450 nm with a ratio of approximately 10:1. Fluorescence peak ratios, like Em 335/Em 450, could prove to be valuable indicators of spore identity for *B. anthracis* since it is part of the *B. cereus* Group.

These data also demonstrated a relationship between taxonomic identity and hydrophobicity. There was a strong correlation between taxonomy and hydrophobicity within the soil isolates ($R^2 = 0.8$) but not between laboratory isolates. Doyle et al. (1984) suggested that in order to isolate spores from vegetative cells, adherence to hydrophobic surfaces may be a valuable tool. The data reported here suggest that in order to isolate spores from vegetative into hydrophobic materials (e.g. hexadecane) will be a good method to help isolate and concentrate spores from complex environmental samples.

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