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Bacterial Spore Detection and Determination by Use of Terbium Dipicolinate Photoluminescence

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A new method to detect bacterial endospores and determine their concentration was demonstrated by the addition of a solution of terbium chloride to a suspension of bacterial endospores. The terbium chloride reacted with the calcium dipicolinate in the spore case to form terbium(III) dipicolinate anion. Solid particles, including residual bacterial particles, were removed by filtering. The photoluminescence from the solution was measured as a function of excitation wavelength, emission wavelength, and bacterial endospore concentration. The photoluminescence from terbium(III) dipicolinate anion in the solution was easily identified.

A new method for detecting bacterial endospores and determining their concentration is presented that could be very useful in many applications. For example, manufacturers could rapidly monitor bioreactors that make endospore suspensions (such as bacterial insecticides). Public health workers could more rapidly monitor indoor environments, water quality, or food quality.[1,2] Paleontologists, who now can detect only viable endospores in rock and soil,[3,4] could detect nonviable endospores.

Bacterial endospores are much more durable than vegetative (i.e., biologically active) bacterial cells. Endospores resist antiseptics, antibiotics, desiccation, and ordinary boiling more than vegetative cells. Bacterial strains are sometimes identified by these types of resistance. Therefore, the new method could be useful in evaluating some antibacterial and diagnostic techniques by measuring the number of endospores independently of the vegetative cells.

Bacterial endospore concentrations are not easy to determine. The main methods of quantification, microscopy and plate culture counting, are slow and tedious.[5] Although previous investigators have studied the intrinsic photoluminescence of bacteria for identification,[6−8] the intrinsic photoluminescence is not specific to bacterial endospores. Our new method is faster than any other method that is specific to bacterial endospores. We here explain and show experimental validation of the new method.

The method of detecting and determining bacterial spores consists of the following three steps. First, terbium chloride (i.e., $TbCl_3$) is added to an aqueous suspension that may contain bacterial endospores. The terbium cation (Tb^{3+}) reacts with calcium dipicolinate (i.e., Ca(dpa)) in any spore case present to form terbium(III) dipicolinate (i.e., $[Tb(dpa)_3]^{3-}$) anion, a chelate. Second, particles (including the residual bacterial particles) are removed from the terbium-treated suspension by filtration. Because $[Tb(dpa)_3]^{3-}$ is soluble, it is easily separated from insoluble concomitants. Third, the photoluminescence of $[Tb(dpa)_3]^{3-}$ is measured. To explain why this method works, we compare Ca(dpa) to $[Tb(dpa)_3]^{3-}$.

Calcium dipicolinate (i.e., Ca(dpa)) is the major component in spore cases of bacterial endospores[2] but is otherwise uncommon. Previous investigators have used the absorbance spectrum of Ca(dpa) to measure bacterial endospore concentrations.[9] For many other compounds, measuring photoluminescence is a more sensitive method for determining concentration than measuring absorbance. However, Ca(dpa) does not generate photoluminescence.

Terbium(III) dipicolinate (i.e., $[Tb(dpa)_3]^{3-}$), in contrast to Ca(dpa), has a very strong and distinctive photoluminescence spectrum. Terbium cation (i.e., Tb^{3+}) reacts with dipicolinate anion (i.e., dpa^{2-}) to form $[Tb(dpa)_3]^{3-}$. The positions of the peaks in the emission spectrum of $[Tb(dpa)_3]^{3-}$ are unchanged from those of Tb^{3+}, and the emission bands of $[Tb(dpa)_3]^{3-}$ are very narrow because of shielding by outer s and p orbital electrons in the terbium atom.[10] At a given concentration and excitation intensity, the photoluminescence intensity of $[Tb(dpa)_3]^{3-}$ is far greater than that of terbium cation, Tb^{3+}, alone.[11]

Previous investigations that used dipicolinic acid to detect Tb^{3+} led to the idea of the new endospore determination method.[12,13] Dipicolinic acid (i.e., H_2dpa) was added to a sample, releasing dipicolinate anions (i.e., dpa^{2-}), which reacted with Tb^{3+} to form $[Tb(dpa)_3]^{3-}$, from which photoluminescence spectra were measured. Because both Ca(dpa) and H_2dpa release dpa^{2-} in solution, we predicted that the Ca(dpa) from bacterial endospores would

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also react with Tb^{3+} to form $[Tb(dpa)_3]^{3-}$, which could be used to detect and determine bacterial endospores.

EXPERIMENTAL SECTION

Sample Preparation. Solutions of both endospore suspensions with $TbCl_3$ and $[Tb(dpa)_3]^{3-}$ without endospores were prepared for comparison. All samples were prepared in 50 mM TRIS buffer solution, which consisted of 6.36 g/L of Trizma·HCl, 1.18 g/L of Trizma·Base (Sigma Chemical Co.), and 4% (v/v) ethanol. The pH of the solution was 7.7, as measured with a pH meter.

Solutions of $TbCl_3$ were prepared by serial dilution. At high concentrations (>1 mM) of $TbCl_3$, a precipitate formed in TRIS buffer solution after 6 h. No precipitation occurred at the small concentrations (<300 μM) of $TbCl_3$ used for our measurements. Therefore, we avoided precipitation by immediately diluting high concentrations of $TbCl_3$. We prepared solutions of $[Tb(dpa)_3]^{3-}$ without bacterial endospores by adding H_2dpa solution to 41 μM $TbCl_3$.

A stock spore suspension of *Bacillus subtilis* was obtained from AMSCO Sterility Products (Apex, NC). The mean population recovery (i.e., concentration of spores in colony forming units (CFU) per milliliter) of this suspension was 6.2×10^9 CFU/mL in 40% (v/v) ethanol.

The effect of filtering terbium-treated endospore suspensions was studied in 200 μM $TbCl_3$. A 0.08-mL volume of the AMSCO suspension was added to 10 mL of 200 μM $TbCl_3$. Spectra of this suspension were measured before and after filtering with Millipore Millex-GV syringe filters (pore size 0.22 μm), which removed insoluble particles. A 200 μM $TbCl_3$ blank and a 50 nM $[Tb(dpa)_3]^{3-}$ solution without endospores were also examined before and after filtering.

Both high-resolution spectra and the calibration curve were studied in 31.4 μM $TbCl_3$. AMSCO spore suspensions (0.0010, 0.0032, 0.0160, 0.080, and 0.40 mL) were dispersed into five 10-mL samples of 31.4 μM $TbCl_3$. Two vials, one with 400 nM $[Tb(dpa)_3]^{3-}$ and another with a 31.4 μM $TbCl_3$ blank, did not contain bacteria. These samples were immediately filtered through a syringe containing Millipore Millex-GV filters (pore size 0.22 μm).

Data Acquisition and Analysis. An excitation spectrum, an emission spectrum, and an absorbance spectrum were taken for each sample. Photoluminescence excitation and emission spectra were measured from each sample with an SLM Model 48000S spectrofluorometer (Spectronics Instruments, Inc., Rochester, NY). A 420-nm long-pass filter was in front of the emission monochromator at all times, to eliminate second-order diffraction of elastically scattered light. All intensities shown in the figures are proportional to the luminescence intensity divided by the excitation intensity. Absorbance spectra were measured with a UV−vis spectrophotometer (Perkin-Elmer Model Lambda 6).

A calibration curve was measured for *B. subtilis* at the peak intensity, which was at 270-nm wavelength excitation and 546-nm wavelength emission. Each data point consisted of an average of five replicates. All measurements were repeated whenever the bias voltage for the emission photomultiplier tube (PMT) was changed. The values shown are corrected for differing PMT bias voltages.

An appropriate wavelength resolution was used for each measurement. To include the entire excitation spectrum of the unfiltered suspension with a few data points, we measured the spectra of the 200 μM $TbCl_3$ solutions with 4-nm wavelength resolution for both excitation and emission monochromators. Because wavelengths longer than 300 nm neither are absorbed nor excite photoluminescence in $[Tb(dpa)_3]^{3-}$, the excitation spectra of filtered suspensions were narrow relative to those of the unfiltered suspensions. Therefore, spectra of the filtered 31.4 μM $TbCl_3$ were measured with 2-nm wavelength resolution for both excitation and emission monochromators. To maximize the signal for the calibration curve, we measured the signal with 4-nm wavelength resolution at the peak.

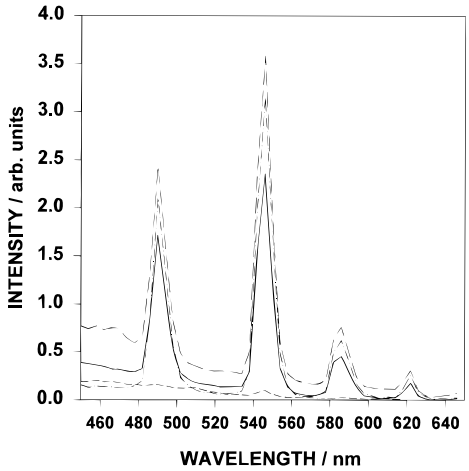

Figure 1. Emission spectra at 270-nm excitation wavelength and 4-nm wavelength resolution for a 0.08-mL *B. subtilis* suspension in 200 μM $TbCl_3$ both before (– –) and after (—) filtering, for 200 μM $TbCl_3$ blank (- - -), and for scaled 50 nM $[Tb(dpa)_3]^{3-}$ (—·—).

RESULTS

The experiments without bacteria confirmed the results of studies done by other researchers:[10,12−14] notably, the calibration curve of $[Tb(dpa)_3]^{3-}$ is linear with concentration, and the absorbance is too small to affect luminescence through inner filtering at the concentrations used in this study. The maximum decadic molar absorptivity of $[Tb(dpa)_3]^{3-}$ was 5050 L/(mol cm) at 271-nm wavelength.

Filtering made the photoluminescence bands of terbium-treated endospore suspensions much narrower. Emission spectra are shown in Figure 1 and excitation spectra in Figure 2 for both filtered and unfiltered samples with 0.08 mL of AMSCO *B. subtilis* suspension and from the 200 μM $TbCl_3$ blank on the same scale. For comparing spectral shapes with the chelate, the figures also show spectra from 50 nM $[Tb(dpa)_3]^{3-}$ at a magnified scale. Because a typical bacterial spore has a radius of about 1 μm, we assume that the filters with a 0.22-μm pore size removed the insoluble parts of endospores from the suspension, which made filtered spectra narrower.

The photoluminescence spectra of $[Tb(dpa)_3]^{3-}$ and the filtered *B. subtilis* suspensions were compared. Emission spectra are shown in Figure 3 and excitation spectra in Figure 4 for a sample with 0.4 mL of AMSCO *B. subtilis* suspension in 10 mL of a solution of 31.4 μM $TbCl_3$, 31.4 μM $TbCl_3$ blank, and 400 nM $[Tb(dpa)_3]^{3-}$ solution without endospores. The photoluminescence intensity of the 0.4-mL AMSCO spore suspension was almost identical to that of 400 nM $[Tb(dpa)_3]^{3-}$ solution.

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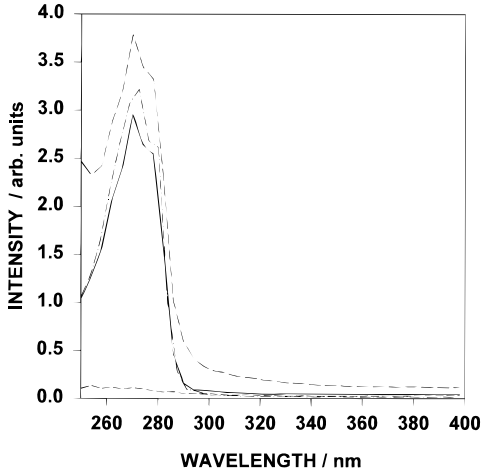

Figure 2. Excitation spectra at 546-nm emission wavelength and 4-nm wavelength resolution for a 0.08-mL *B. subtilis* suspension in 200 μM $TbCl_3$ both before (– –) and after (—) filtering, for 200 μM $TbCl_3$ blank (- - -), and for scaled 50 nM $[Tb(dpa)_3]^{3-}$ (—•—).

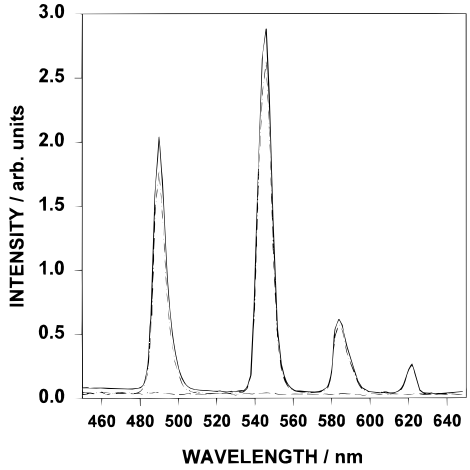

Figure 3. Emission spectra at 270-nm excitation wavelength and 2-nm wavelength resolution for a filtered 0.40-mL *B. subtilis* suspension in 31.4 μM $TbCl_3$ (—), for 31.4 μM $TbCl_3$ blank (- - -), and for 400 nM $[Tb(dpa)_3]^{3-}$ (—•—).

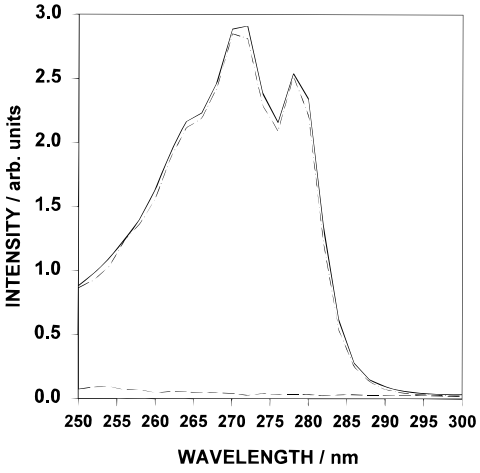

Figure 4. Excitation spectra at 546-nm emission wavelength and 2-nm wavelength resolution for a filtered 0.40-mL *B. subtilis* suspension in 31.4 μM $TbCl_3$ (—), for 31.4 μM $TbCl_3$ blank (- - -), and for 400 nM $[Tb(dpa)_3]^{3-}$ (—•—).

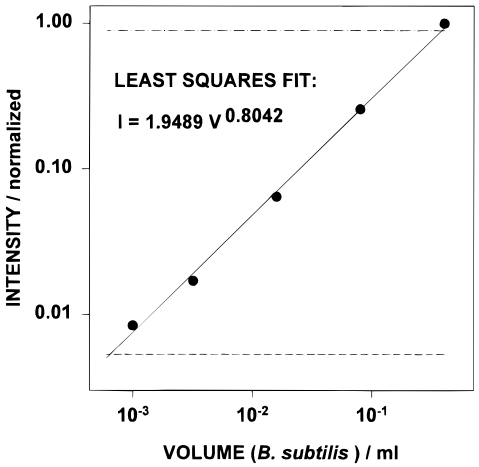

Figure 5. Calibration curve data (●) and power law fit (—) of filtered *B. subtilis* suspensions for 31.4 μM $TbCl_3$, with 31.4 μM $TbCl_3$ blank (- - -) and 400 nM $[Tb(dpa)_3]^{3-}$ (—•—) shown for comparison.

The dependence of luminescence intensity, *I*, on the volume, *V*, of AMSCO spore suspension is shown in Figure 5. The percent standard deviation of each data point was below 2%, so the error bars are smaller than the symbols. All luminescence intensities were normalized to that of the sample with 0.4-mL AMSCO suspension. The volume was not converted to CFU in this figure because of the well-known difficulty of interpreting CFU. Interpreting CFU is often a problem when standard microbiology methods are used because of nonviable spores and spore aggregation.[1,2,5] Figure 5 also shows the luminescence intensity of the 31.4 μM $TbCl_3$ blank and the 400 nM $[Tb(dpa)_3]^{3-}$ for comparison. The intensity of the most concentrated spore suspension was nearly identical to that of 400 nM $[Tb(dpa)_3]^{3-}$.

A nonlinear least-squares fit to the data using a power law formula is shown in Figure 5, showing that the luminescence intensity was equal to the volume of AMSCO suspension to the power of 0.8042 and a coefficient of 1.9489 within 0.1% in both parameters. The correlation coefficient, r^2, was equal to 0.9997. Therefore, the calibration curve follows a power law of 0.8042 instead of 1.0.

The limit of detection (LOD) was calculated from the calibration curve at a signal equal to the luminescence intensity of the blank plus 3 times the standard deviation of the blank. The LOD was 0.00073 mL of AMSCO suspension, which was equivalent to 4.4×10^5 CFU/mL of 31.4 μM $TbCl_3$. The blank luminescence was greater than the standard deviation, showing that the major concomitant was Tb^{3+}.

DISCUSSION

The spectra show that bacterial spores react with $TbCl_3$ to form $[Tb(dpa)_3]^{3-}$. The filtered samples of terbium-treated spores have spectral shapes closely resembling those of $[Tb(dpa)_3]^{3-}$. However, the unfiltered terbium-treated spore samples had a broad-band component similar to that of endospores not treated with terbium. Because this broad-band component disappears after filtering, it may be the intrinsic photoluminescence of bacteria described by other researchers.[6–8]

The luminescence spectra from terbium-treated endospores are more distinctive than those of untreated bacteria. Unlike $[Tb(dpa)_3]^{3-}$, the luminescence spectra from bacteria untreated with Tb^{3+} are very broad and devoid of line structure.[6,7] Two peaks (at 270 and 278 nm) show in the excitation spectrum of terbium-treated spores, while only one peak (at 280 nm) shows in the excitation spectrum of bacteria untreated with terbium. Four

lines (at 490, 546, 586, and 622 nm) show in the emission spectrum of terbium-treated endospores, while only one strong band (at 340 nm), which is very broad, shows in the emission spectrum of untreated bacteria.

The power law exponent, 0.8042, in the calibration curve of the endospores was completely unexpected. One possible explanation is the presence of a substance in the AMSCO spore suspension that quenches photoluminescence. Some substances could quench photoluminescence of $[Tb(dpa)_3]^{3-}$ by competing with Tb^{3+} for the dipicolinate anion.

Calcium could be the major quenching agent. We describe one possible model for a sublinear power law with calcium as the quencher. We assume that the following reaction dominates the equilibrium of this system:[13]

$$Tb^{3+} + 3Ca(dpa) \rightleftarrows [Tb(dpa)_3]^{3-} + 3Ca^{2+}$$

There was probably a large excess of Tb^{3+} in this experiment, because the luminescence of terbium-treated endospore suspensions was smaller than that of 400 nM $[Tb(dpa)_3]^{3-}$ and the $TbCl_3$ concentration was much higher than 400 nM. If there is an excess of Tb^{3+}, if all calcium and dipicolinate ions come entirely from Ca(dpa), if the photoluminescence comes entirely from $[Tb(dpa)_3]^{3-}$, and if the initial concentration of Ca(dpa) is much greater than the equilibrium concentration of Ca^{2+}, one can show that the power law exponent would be 0.75 instead of 1.0. The measured power law, 0.8042, is close to 0.75. The lipid component of the bacterial endospores could also affect the calibration curve. Phospholipid vesicles have been shown to affect calibration curves of $[Tb(dpa)_3]^{3-}$.[15]

A lower LOD would result from removal of Tb^{3+} photoluminescence, although this was not done in the present study. The LOD previously calculated, 4.4×10^5 CFU/mL, is valid only for 31.4 μM $TbCl_3$. Therefore, one can decrease the LOD by decreasing the background concentration of $TbCl_3$. Alternatively, if there is an excess of $TbCl_3$, the intensity of Tb^{3+} emission could be measured from the blank and analytically subtracted, resulting in a much lower LOD.

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