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Real-Time Detection of Kinetic Germination and Heterogeneity of Single *Bacillus* Spores by Laser Tweezers Raman Spectroscopy

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Germination is the process by which a dormant spore returns to its vegetative state when exposed to suitable conditions. We report on the real-time detection of kinetic germination and heterogeneity of single *Bacillus thuringiensis* spores in an aqueous solution by monitoring the calcium dipicolinate (CaDPA) biomarker with laser tweezers Raman spectroscopy (LTRS). A single *B. thuringiensis* spore was optically trapped in a focused laser beam, and its Raman spectra were recorded sequentially in time after exposure to a nutrient-rich medium, so that the CaDPA amount inside the trapped spore was monitored during the dynamic germination process. The CaDPA content in an individual spore was observed to remain almost constant in the first period and then decrease very rapidly due to its release into the medium (within ~2 min). The time-to-germination (t_{germ}), defined as the time required for the CaDPA band intensity to decrease to the midpoint from its initial value, was found to be stochastic for individual spores with a typical value of ~30 min under the experimental conditions. The distribution of the time-to-germination was measured from a time lapse measurement of a population of spores. The results demonstrated that LTRS can be used to noninvasively detect the kinetic germination process at the single-cell level and explore cellular heterogeneity.

The ability to monitor biological dynamics of individual cells and explore cellular heterogeneity is of particular interest to single-cell microbiology.[1−3] Traditionally, information on how cells respond to the environment, interact with each other, or undergo complex biological processes was obtained from population-level studies. However, properties such as cell viability, biochemical composition, or activation are the intrinsic states or properties of each individual cell.[1] Bulk-scale measurements report only average values for the population and are not capable of determining the contributions of individual heterogeneous cells. Cellular heterogeneity within a microbial population originates from genetic differences, biochemical differences, physiological differences, and behavioral differences. Several analytical tools and technologies including fluorescence, cytometry, scanning probe microscopy, Raman microscopy, and micromanipulation have been used to resolve individual cellular differences.[1−3] This paper studies cellular heterogeneity of kinetic germination of single *Bacillus* spores using laser tweezers Raman spectroscopy (LTRS).

The study of cellular heterogeneity in bacterial spores and their germination process is also of importance for the detection of biological attacks and food safety.[4−7] Bacterial spores are resting structures formed for survival under adverse environmental conditions. Germination is the process by which a dormant spore returns to its vegetative state when exposed to suitable conditions. The transformation of a dormant spore into a vegetative cell is a very important step in the pathogenesis of the bacterium. The spore germination is related to a series of biophysical processes. During germination, the spore inner membrane becomes permeable, ion fluxes resume, and the spore gets rehydrated; the calcium ions and dipicolinic acid are released from inside the spore. The spore cortex and coat layers degrade; the spore restores its metabolism. The release of calcium dipicolinate (CaDPA) is a major step in spore germination.[6,7] Recently, CaDPA has been used as a useful biomarker for the detection of bacillus spores.[4,5] In this approach, CaDPA was extracted from the spores and then identified by either surface-enhanced Raman spectroscopy or FAST coherent anti-Stokes Raman spectroscopy. CaDPA biomarker can also be detected directly inside single bacterial spores using Raman spectroscopy, without the need of extraction from living cells.[8−10] However, the CaDPA content inside a spore can change dynamically during the germination process and thus

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(1) Brehm-Stecher, B. F.; Johnson, E. A. *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 538−559.

(2) Shapiro, H. M. *J. Microbiol. Methods* **2000**, *42*, 3−16.

(3) Davey, H. M.; Kell, D. B. *Microbiol. Rev.* **1996**, *60*, 641−696.

(4) Zhang, X.; Young, M. A.; Lyandres, O.; Van Duyne, R. P. *J. Am. Chem. Soc.* **2005**, *127*, 4484−4489.

(5) Pestov, D. M.; Zhi, M.; Sariyanni, Z.; Kalugin, N. G.; Kolomenskii, A. A.; Murawski, R.; Paulus, G. G.; Sautenkov, V. A.; Schuessler, H.; Sokolov, A. V.; Welch, G. R.; Rostovtsev, Y. V.; Siebert, T.; Akimov, D. A.; Graefe, S.; Kiefer, W.; Scully, M. O. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 14976−14981.

(6) Torrents, E.; Sahlin, M.; Biglino, D.; Graslund, A.; Sjoberg, B. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 17946−17951.

(7) Stringer, S. C.; Webb, M. D.; George, S. M.; Pin, C. Peck, M. W. *Appl. Environ. Microbiol.* **2005**, *71*, 4998−5003.

(8) Manoharan, R.; Ghiamati, E.; Dalterio, R. A.; Britton, K. A.; Nelson, W. H.; Sperry, J. F. *J. Microbiol. Methods* **1990**, *11*, 1−15.

(9) Alexander, T. A.; Pellegrino, P. M.; Gillespie, J. B. *Appl. Spectrosc.* **2003**, *11*, 1340−1345.

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may affect the bacterial identification that is based on the detection of the CaDPA marker.

The germination of individual spores is kinetic and heterogeneous.[11−16] Hashmoto and co-workers[11] studied the germination of single spores by use of a microscope photometer and revealed the biphasic nature of spore germination. A kinetic model for bacterial spore germination was proposed by Woese et al.[12] Recently, Stringer et al.[7] measured the distribution of times required for germination and outgrowth from spores of *Clostridium botulinum* using phase-contrast microscopy. Zaman et al.[16] studied the change of outer spore membrane and the cortical layers of *Bacillus anthracis* spores during germination by atomic force microcopy and transmission electron microscopy. The kinetic germination of individual spores was usually observed with phase-contrast microscopy,[7,11−15] based on the fact that spore germination is accompanied by a change in the refraction. The light refractile (phase bright) corresponds to a dormant spore and nonrefractile (phase dark) corresponds to a germinated spore. The time-to-germination of an individual spore was defined as the midpoint of the intensity from phase bright to phase dark images.[7] Although the change in refractility had been considered to be related to the release of the CaDPA to the environment and loss of heat resistance,[11−15] phase-contrast microscopy cannot provide direct molecular information.

The release of CaDPA components during spore germination has been measured with various spectroscopic bioassays in bulk scale.[17−20] Scott and Ellar[17] studied the release of CaDPA from *Bacillus megaterium* during L-alanine triggering of germination using UV absorbance spectroscopy. Cheung et al.[19] monitored CaDPA components of *Bacillus subtilis* endospores during germination using Fourier transform infrared spectroscopy. In these bioassay studies, the measured CaDPA was the average value over a large population of spores. Since the germination time is heterogeneous among individual spores, the fast CaDPA release process of an individual spore was masked by population average and has not been directly observed yet.

In this paper, we report on real-time observation of kinetic germination of single *Bacillus thuringiensis* spores (a harmless simulant of *B. anthracis*) by LTRS, based on the monitoring CaDPA biomarker. Heterogeneity in the kinetic germination of an individual spore is studied by the measurement of time-to-germination distribution. The LTRS is the combination of optical tweezers and Raman microspectroscopy.[21,22]The LTRS technique allows capture of a single spore in the focus of the laser beam and then collecting its Raman spectra noninvasively. From Raman spectra, the dynamic change in biomolecules such as proteins, nucleic acids, lipids, and carbohydrates as well as CaDPA components inside a living cell can be selectively monitored. The LTRS has been applied for the detection of bacterial spores,[9,10] discrimination and sorting of microorganisms,[23,24] and analysis of human lipoproteins[25] and chromosomes.[26] Recently, LTRS has been applied to monitor the dynamic biological processes of single cells.[27−29]

In this experiment, a single *B. thuringiensis* spore was optically trapped in a focused laser beam and its Raman spectra were recorded sequentially in time after exposure to an aqueous nutrient-rich medium, so that the CaDPA amount inside the individual spore was monitored during the nutrient-triggered germination process. Since a near-infrared laser beam at 785 nm with very low power (5 mW) was used for both optical trapping and Raman excitation, the possible photodamage to the spore and the laser-induced effect on the kinetic spore germination were minimized due to the low absorption in NIR region.[30] *B. thuringiensis (Bt)*, a Gram-positive, endospore-forming bacterial species, which is used as insecticides, was used as a model system in the germination study. *Bt* spores can produce toxic crystals that are pathogenic to insects, but harmless to human beings. The *Bt* spore has some physiological similarities with the *B. anthracis* spore.

MATERIALS AND METHODS

Bacterial Strain and Sample Preparation. The wild-type strain of *B. thuringiensis* spore powder (Ward's Natural Science Inc., Rochester, NY) was suspended in fresh Tryptic Soy Broth (TSB; Fisher Scientific Inc., Suwanee, GA, No. 211825, prepared by adding 30 g in 1 L of water) and then incubated with shaking (37 °C, overnight) for the initial cell culture. The diluted cell culture was streaked on a sterile Tryptic Soy Agar plate (Fisher Scientific Inc., No. 236950) and incubated for 7 days at 30 °C. The majority of cells in a single colony on the TSA plate were observed to be in the spore state and the spore plate was stored at 4 °C prior to use.

Laser Tweezers Raman Spectroscopy. The experimental setup was similar to the previous papers.[22,23] A laser beam from a wavelength-stabilized diode laser at 785 nm is circularized with a prism pair, spatially filtered, and then introduced in an inverted differential interference contrast (DIC) microscope (Nikon TE2000) equipped with an objective (100×, NA = 1.30) to form a single-beam optical trap. A bacterial cell in an aqueous medium can be

(10) Chan, J. W.; Esposiko, A. P.; Talley, C. E.; Hollars, C. W.; Lane, S. M.; Huser, T. *Anal. Chem.* **2004**, *76*, 599−603.
(11) Hashimoto, T.; Frieen, W. R.; Conti, S. F. *J. Bacteriol.* **1969**, *98*, 1011−1020.
(12) Woese, C. R.; Vary, J. C.; Halvorson, H. O. *Biochemistry* **1968**, *59*, 869−875.
(13) Moir, A.; Corfe, B. M.; Behravan, J. *CMLS, Cell. Mol. Life Sci.* **2002**, *59*, 403−409.
(14) Caipo, M. L.; Duffy, S.; Zhao, L.; Schaffner, D. W. *J. Appl. Microbiol.* **2002**, *92*, 879−884.
(15) Thrane, C.; Olsson, S.; Wolstrup, J.; Sorensen, J. *J. Appl. Microbiol.* **2000**, *89*, 595−598.
(16) Zaman, M. S.; Goyal, A.; Dubey, G. P.; Gupta, P.; Chandra, H.; Das, T. K.; Ganguli, M.; Singh, Y. *Microsc. Res. Tech.* **2005**, *66*, 307−311.
(17) Scott, L. R.; Ellar, D. J. *J. Bacteriol.* **1978**, *135*, 133−137.
(18) Moir, A. In *Biology of Bacilli*; Doi, R. H., McGloughlin, M., Eds.; Butterworth-Heinemann: London, 1992; pp 23−38.
(19) Cheung, H. Y.; Cui, J.; Sun, S. Q. *Microbiology* **1999**, *145*, 1043−1048.
(20) Kort, R.; O'Brien, A. C.; van Stokkum, I. H. M.; Oomes, S. J. C. M.; Crielaard, W.; Hellingwerf, K. J.; Brul, S. *Appl. Environ. Microb.* **2005**, *71*, 3556−3564.
(21) Xie, C.; Dinno, M. A.; Li, Y. Q. *Opt. Lett.* **2002**, *27*, 249−251.
(22) Xie, C.; Li, Y. Q. *J. Appl. Phys* **2003**, *93*, 2982−2986.
(23) Xie, C.; Mace, J.; Dinno, M. A.; Li, Y. Q.; Tang, W.; Newton, R. J.; Gemperline, P. J. *Anal. Chem.* **2005**, *77*, 4390−4397.
(24) Xie, C.; Chen, D.; Li, Y. Q. *Opt. Lett.* **2005**, *30*, 1800−1802.
(25) Chan, J. W.; Motton, D.; Rutledge, J. C.; Keim, N. L.; Huser, T. *Anal. Chem.* **2005**, *77*, 5870−5876.
(26) Ojeda, J. F.; Xie, C. A.; Li, Y. Q.; Bertrand, F. E.; Wiley: J.; McConnell, T. J. *Opt. Expression* **2006**, *14*, 5386-5393.
(27) Xie, C.; Li, Y. Q.; Tang, W.; Newton, R. J. *J. Appl. Phys.* **2003**, *94*, 6138−6142.
(28) Ajito, K.; Han, C., Torimitsu, K. *Anal. Chem.* **2004**, *76*, 2506−2510.
(29) Singh, G. P.; Creely, C. M.; Volpe, G.; Grotsch, H.; Petrov, D. *Anal. Chem.* **2005**, *77*, 2564−2568.
(30) Neuman, K. C.; Chadd, E. H.; Liou, F.; Bergman, K.; Block, S. M. *Biophys. J.* **1999**, *77*, 2856−2863.

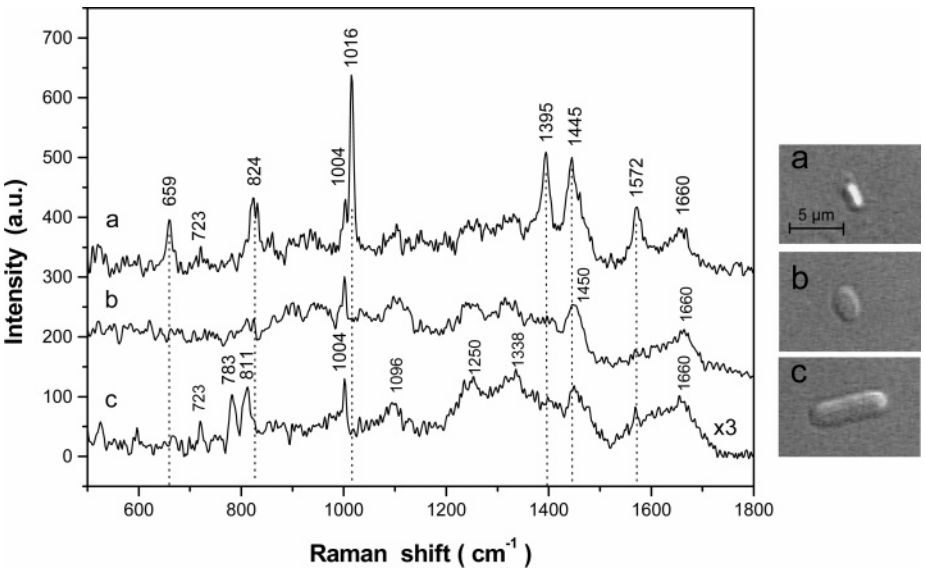

Figure 1. Raman spectra and images of a *B. thuringiensis* spore (curve a), a germinated spore (curve b), and a vegetative (dividing) cell (curve c). The intensity of curve c is magnified by a factor of 3 for display. The positions of the Raman bands of CaDPA at 1572, 1445, 1395, 1016, 824, and 659 cm^{-1} are marked.

trapped ~10 μm above the bottom coverslip with the gradient force yielded by the focused beam. The same laser beam excites Raman scattering from the trapped particle. The collimated backward Raman scattering light, after spatial and spectral filtering, is focused onto the entrance slit of a spectrograph and detected by a liquid nitrogen-cooled, charge-coupled detector (Symphony CCD, Jobin Yvon). The image of the trapped particle can be viewed through a video camera system illuminated with a green-filtered lamp. The Raman spectra were recorded in the "fingerprint" range from 500 to 1800 cm^{-1} with a spectral resolution of ~6 cm^{-1}. The background spectrum was taken under the same acquisition conditions without the cell in the trap and subtracted from the collected spectra of individual cells. The subtracted spectra were then smoothed using the Savitzky−Golay filter method, and peak heights at particular wavenumbers were read out. The Brownian motion and cell motility make the trapped spores randomly rotate inside the laser beam with a small amplitude and with a much shorter time scale than the Raman acquisition time and thus do not affect Raman data acquisition.

Measurement of Kinetic Germination of Single Spores. A small amount of *Bt* spores from a single colony of the stock plate were suspended into 3 mL of preheated TSB medium and then quickly loaded into a microscope sample holder that was kept at 37 °C during the whole process of measurement using a laboratory temperature control unit. After loading the spore sample, a single spore in TSB nutrient medium was randomly selected and kept captured during the measurement so that its kinetic germination process was recorded in real time. Raman spectra of the trapped spore were acquired continually with a laser power of 5 mW and a CCD acquisition time of 60 s until germination was completed. At the same time of each Raman acquisition, we recorded the DIC microscopic images of the trapped spore with a video imaging card (Coreco Imaging) so that the brightness of the spore images can be quantified. The image of a dormant spore is very bright and becomes dark when it is germinated. This property allowed quick selection of a dormant spore in the trap once the spore sample was loaded. The time required for the procedures of exposing stocked spore sample to the TSB nutrient medium, loading the sample into the preheated sample holder, and trapping a selected dormant spore was 1−2 min. After the completion of recording the germination process of the trapped spore within ~60 min, a new spore sample from the same colony of the stocked agar plate was prepared and loaded so that the measurement on the next individual spore was started. The above procedure was repeated for the observation of each spore. From single-cell Raman spectra, the CaDPA concentration inside the cell can be determined from the intensities at the peaks of 1016, 1395, and 1572 cm^{-1}. The time-to-germination (t_{germ}) was then defined as the time required for the decrease of the CaDPA band intensity to the midpoint of its initial value.

Measurement of Germination of Spore Population. Raman spectra of a spore population up to 30 cells were measured every minute after the spores were exposed to the nutrient medium for incubation. For this purpose, a total of 51 tubes labeled, 0−50 min, were used and each tube contained 4 mL of TSB medium preheated to 37 °C. Spore sample from the stocked TSA plate was first suspended in a tube containing 10 mL of TSB medium precooled at 4 °C, followed by shaking for a few seconds. Then, ~100 μL of the spore suspension was transferred into each labeled TSB tube and placed into a shaking incubator at 37 °C. At a specific incubation time, a labeled tube of cell culture was removed from the incubator and immediately stored in a 4 °C refrigerator before use. The maximum incubation time was 50 min. Measurement on the cell culture of each tube was done by transferring 100 μL of the stored cold culture into a temperature-controlled microscope sample holder kept at 4 °C, randomly capturing a cell, and acquiring its Raman spectrum with a laser power of 30 mW and an acquisition time of 20 s. Up to 30 spores were randomly selected for measurement for each labeled tube of cell culture (with a specific incubation time). The average Raman spectra for each incubation time can be calculated and the population-averaged CaDPA amount inside the spore can be obtained as a function of the incubation time.

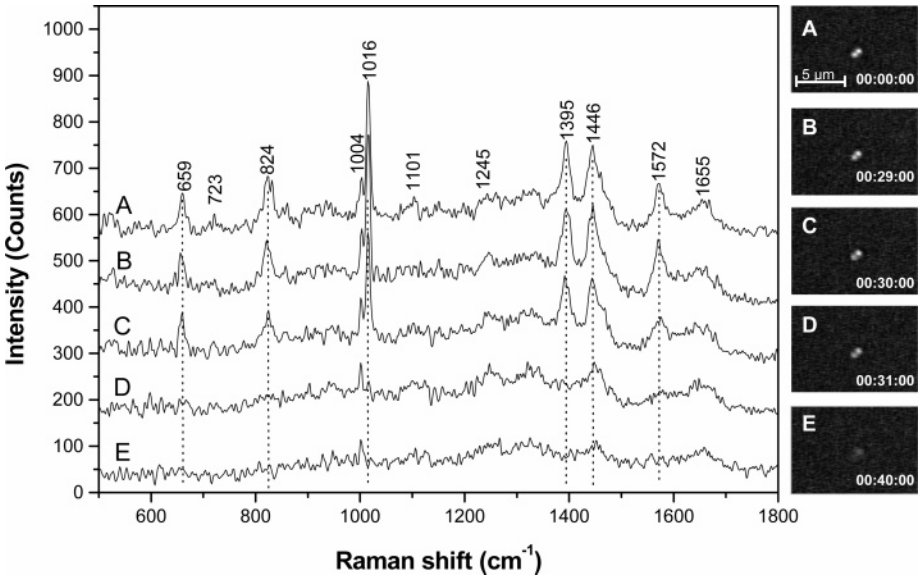

Figure 2. Time-lapse Raman spectra of a single trapped *B. thuringiensis* spore after exposure to the TSB growth medium and the corresponding DIC images. Curve A and image A are for 0 min from the time when the spore was captured in the optical trap, B for 29 min, C for 30 min, D for 31min, and E for 40 min. The time from exposing the spore to the medium to capturing the spore for the start of Raman measurement was less than 2 min. λ_{ex} = 785 nm, P_{ex} = 5 mW, and acquisition time 60 s.

The distribution of the time-to-germination $p(\tau)$ can be derived from the population measurement. Based on the existence or disappearance of the CaDPA bands in single-cell Raman spectra, a detected cell can be classified as ungerminated spore or germinated spore. For each incubation time, the number of ungerminated spores was counted, denoted by $N(t)$, and the ratio $r(t) = N(t)/N_0$ was obtained as the probability of a spore remaining in the spore state by the time t, where N_0 was the total number of the measured cells. Thus, the probability of a spore that has been germinated by time t after exposure to the nutrient medium is given by

$$1 - r(t) = \int_0^t p(\tau)\, \mathrm{d}\tau \qquad (1)$$

where $p(\tau)$ is the probability density function (or distribution function) of a spore whose time-to-germination t_{germ} is equal to τ. From eq 1, the distribution function can be obtained from the measured data by taking the first-order derivative,

$$p(t) = -\,\mathrm{d}r(t)/\mathrm{d}t \qquad (2)$$

RESULTS AND DISCUSSION

Raman Spectra of Single Dormant Spore, Germinated Spore, and Vegetative Cell. Figure 1 shows the typical Raman spectra from a single dormant spore, a germinated spore, and a vegetative (dividing) cell, together with their bright-field microscopic images. Strong Raman bands at 1572, 1445, 1395, and 1016 cm^{-1} and weak vibrations at 824 and 659 cm^{-1} were assigned to CaDPA component of the spore.[4,10] The Raman band at 1004 cm^{-1} was assigned to phenylalanine. Bands at 1250 and 1660 cm^{-1} were assigned to amide III and I vibrations of proteins, respectively.[23] Bands at 783, 811, and 1096 cm^{-1} in bacterial spectra were assigned to nucleic acids.[23] The strong intensity of Raman bands at 1016, 1395, and 1572 cm^{-1} indicates that the concentration of CaDPA inside the spore is high and easily excited in near-infrared wavelengths. Therefore, these bands can be used as the biomarker of CaDPA in *Bacillus* spores. However, as the spore germinates, grows, and divides in nutrient medium, the CaDPA marker disappears in the single-cell Raman spectra (curves b and c). This makes the use of Raman spectroscopy for the real-time monitoring of kinetic germination (or growth cycle) process of single spores possible.

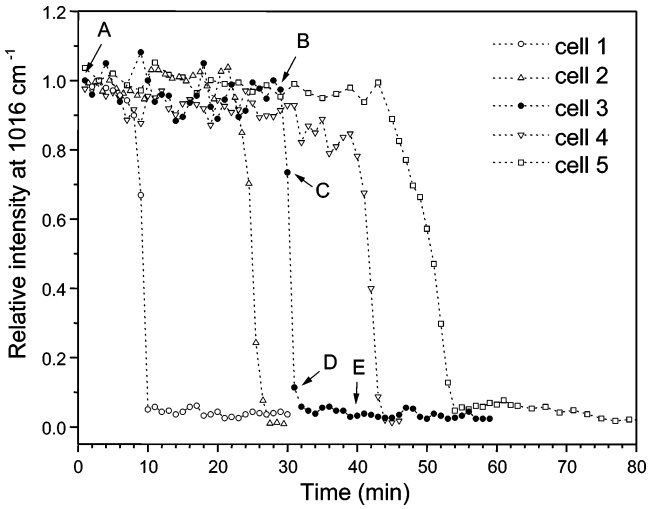

Figure 3. Relative intensities of the 1016-cm^{-1} CaDPA band of five individual *Bt* spores as a function of the incubation time. The intensity at each time point was normalized to that at 0 min. In particular, for cell 3, Raman spectra at the time points A, B, C, D, and E correspond to the spectra A−E given in Figure 2. For all measurements, λ_{ex} = 785 nm, P_{ex} = 5 mW, and acquisition time 60 s.

Kinetic Germination of Single Spores. Figure 2 shows the real-time Raman spectra of a single trapped *B. thuringiensis* spore (cell 3 in Figure 3) after exposure to the TSB growth medium and the corresponding DIC images. In this experiment, a single spore in TSB nutrient medium was randomly selected and trapped in the laser beam, and both its Raman spectra and images were

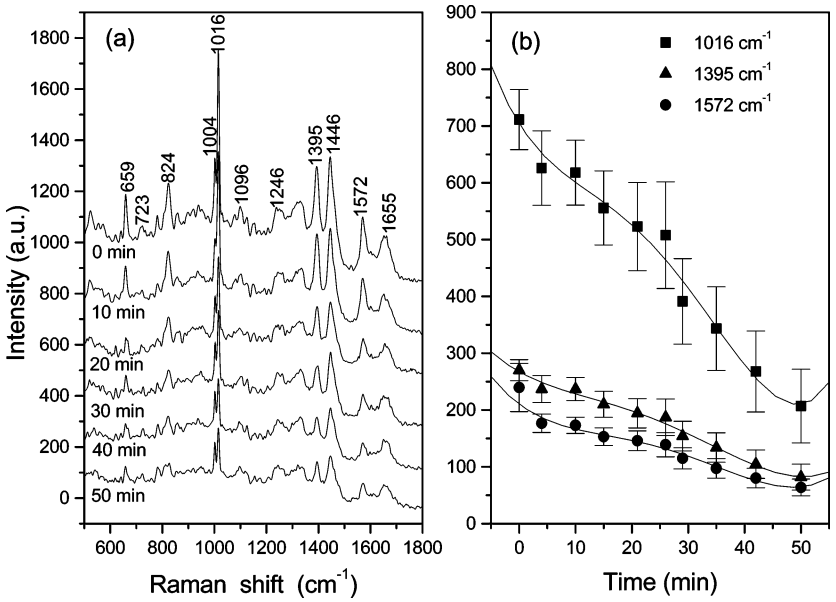

Figure 4. (a) Average spectra of a population of 30 *Bt* spores at different incubation times of 0, 10, 20, 30, 40, and 50 min. (b) Population-averaged intensities of the CaDPA Raman bands at 1016, 1395, and 1572 cm^{-1} as a function of incubation time. The data were fitted with the fourth-order polynomial functions (solid lines). For these measurements, λ_{ex} = 785 nm, P_{ex} = 30 mW, and acquisition time 20 s.

recorded every minute. Although the DIC images show two bright spots in Figure 2, a single spore in the laser trap was verified by its bright-field image or by observing the spore motion after blocking the trap beam. The time required for the procedure from exposing the spore to the medium to capturing the spore for the start of Raman measurement was less than 2 min. The wavelength of the laser excitation was 785 nm, the power was 5 mW, and the acquisition time for each spectrum was 1 min. The results indicated that the CaDPA content (Raman bands at 1016, 1395, and 1572 cm^{-1}) inside this spore experienced two phases: it remained almost constant in the first phase (from 0 to 29 min) and then decreased very rapidly due to its release into the medium in the second phase. The release was completed rapidly within 2 min (from 29 to 31 min). The time-to-germination for this spore was found to be 30 min. The brightness of the cell images observed from the DIC microscopy (Figure 2) confirmed the rapid change in the refractility of the trapped spore. The cell image was bright before germination (from images A to C) and became dark after germination (from images D and E). However, the intensity decrease in cell image is not the direct evidence of CaDPA loss until it is confirmed by Raman spectrum. Actually, as the intensity of CaDPA bands decreased by a factor of 90% (from A to D), the intensity in cell image only decreased by 50%. It took much longer for the residual refractility to disappear (from D to E), indicating that a different process might be involved, which could be a further degradation of spore cortex by enzymes, with eventual hydration of the vital spore core. This observation was consistent with the previous report.[11]

The time-to-germination for individual spores could change from cell to cell and is randomly distributed. Figure 3 shows relative intensities of the 1016-cm^{-1} band for five individual *Bt* spores as a function of the incubation time. The intensity at each time point was normalized to the original value at 0 min. In particular, for cell 3, Raman spectra at time points A, B, C, D, and E correspond to the spectra A−E given in Figure 2. The result shows that each spore completes the decrease in the intensity of the 1016-cm^{-1} CaDPA band rapidly while the time-to-germination varies from cell to cell. For example, t_{germ} is 9 min for cell 1, 25 min for cell 2, 30 min for cell 3, 42 min for cell 4, and 50 min for cell 5 after exposure to the nutrient medium under the same experimental conditions. The result also shows the trend of slow decrease in 1016-cm^{-1} CaDPA band with time for all cells before the fast decrease.

As a control experiment, we repeated the above measurements by exposing *B. thuringiensis* spores to deionized water at 37 °C, rather than to the TSB growth medium. The CaDPA band of an optically trapped spore was observed to remain almost the same height, and no spore germination was observed up to 60 min (not shown in Figure 3). This comparison indicates that the observation of CaDPA release from an optically trapped spore in the TSB medium is due to nutrient-triggered germination, rather than the effect of a low-power near-infrared trapping laser (5mW at 785 nm).

Germination of a Spore Population. Raman spectra of a spore population up to 30 cells were measured at a number of times after the spores were exposed to the TSB nutrient medium for incubation. Figure 4a shows the average Raman spectra of 30 randomly selected spores at different incubation times. Figure 4b plots the average intensities and the standard deviations of the CaDPA bands at 1016, 1395, and 1572 cm^{-1} as a function of incubation time, in which the lines were the fourth-order polynomial fits to the data. The results showed that the average CaDPA amount in a population of spores gradually decreased as incubation time increases. The rapid decrease process of CaDPA component in the germination of individual spores (see Figure 3) was not shown in the population-averaged Raman spectra. This means that the fast CaDPA release feature in kinetic germination of individual spores was masked and cannot be seen in the population measurement. Because the time-to-germination for individual spores change from cell to cell, the population-averaged intensities of CaDPA bands at 1016, 1395, and 1572 cm^{-1} no longer show a sudden decrease at specific incubation time.

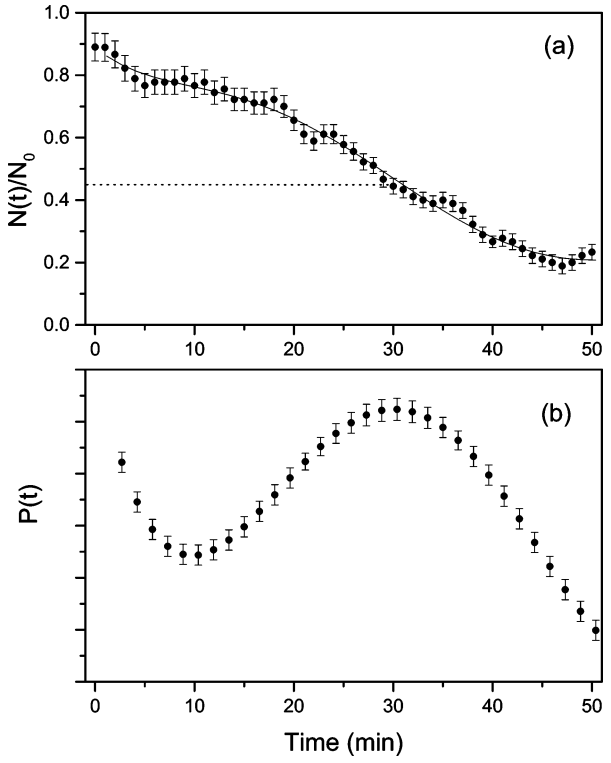

Figure 5. (a) Percentage of ungerminated spores versus incubation time *t*. The solid line is the fourth-order polynomial fit to the data. (b) The derived probability distribution of the time-to-germination of individual spores.

The distribution of the time-to-germination $p(\tau)$ was obtained from the population measurement. Figure 5a shows the ratio $r(t) = N(t)/N_0$ of the ungerminated spore number $N(t)$ that remain in the spore state by the incubation time t to the measured spore number N_0. The ungerminated spores were characterized by the existence of CaDPA bands at 1016, 1395, and 1572 cm^{-1} in single-cell Raman spectra. The resolution of incubation time was 1 min. A fourth-order polynomial function was fitted to the measured data points (the smooth line). By taking the first-order derivative from eq 2, Figure 5b shows the probability distribution of the time-to-germination $p(\tau)$. The most probable spore germination time from the population measurement was ~30 min under the experimental conditions, which was consistent with the observation in the single-spore measurement shown in Figure 3. Apparently, population measurement of spore germination is complimentary to single-spore measurement.

CONCLUSION

We have applied LTRS for the real-time detection of kinetic germination of single *Bacillus* endospores in aqueous nutrient medium and for the study of cellular heterogeneity among individual spores. We monitored the CaDPA amount inside single *B. thuringiensis* endospores during the germination process based on Raman spectra excited with a near-infrared laser beam. We found that the CaDPA content in an individual spore was almost constant in the first period and then decreased very rapidly (within ~2 min) due to its release into the medium. This rapid CaDPA release property in the kinetic germination of individual spores was masked in the population-level measurement. The time-to-germination for individual spores was found to be stochastic with a typical value of ~30 min under the experimental conditions. The distribution of the time-to-germination was measured from a time-lapse measurement of a population of spores. The results demonstrate that LTRS is a reagentless, noninvasive method to monitor kinetic spore germination and CaDPA biomarker at the single-cell level. To the best of our knowledge, this is the first report on a direct observation of cellular heterogeneity in single-spore germination by the use of LTRS technique.

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