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Characterization of Bacterial Spore Germination Using Integrated Phase Contrast Microscopy, Raman Spectroscopy, and Optical Tweezers

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We present a methodology that combines external phase contrast microscopy, Raman spectroscopy, and optical tweezers to monitor a variety of changes during the germination of single *Bacillus cereus* spores in both nutrient (L-alanine) and non-nutrient (Ca-dipicolinic acid (DPA)) germinants with a temporal resolution of ~2 s. Phase contrast microscopy assesses changes in refractility of individual spores during germination, while Raman spectroscopy gives information on changes in spore-specific molecules. The results obtained include (1) the brightness of the phase contrast image of an individual dormant spore is proportional to the level of CaDPA in that spore; (2) the end of the first Stage of germination, revealed as the end of the rapid drop in spore refractility by phase contrast microscopy, precisely corresponds to the completion of the release of CaDPA as revealed by Raman spectroscopy; and (3) the correspondence between the rapid drop in spore refractility and complete CaDPA release was observed not only for spores germinating in the well-controlled environment of an optical trap but also for spores germinating when adhered on a microscope coverslip. Using this latter method, we also simultaneously characterized the distribution of the time-to-complete-CaDPA release ($T_{release}$) of hundreds of individual *B. cereus* spores germinating with both saturating and subsaturating concentrations of L-alanine and with CaDPA.

Bacterial spores of *Bacillus* species are metabolically dormant, very resistant to a variety of harsh conditions, and can survive for many years.[1,2] However, spores can rapidly return to active growth through germination followed by outgrowth, with germination triggered by specific nutrients and some non-nutrient agents.[2−4] Nutrient germinants bind to specific receptors located in the spore's inner membrane, triggering the release of spore small molecules, most notably the large depot (~10% of spore dry wt) of pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)).[2,3] DPA is located exclusively in the spore's central region or core as a 1:1 chelate with divalent cations, predominantly Ca^{2+} (CaDPA) that comprises ~25% of core dry weight, and the core also has quite low levels of water (25−50% of wet wt, depending on the species).[5] Because of its high solid/water ratio, the spore core has a very high refractive index. Release of small molecules, in particular CaDPA, and their replacement by water comprise Stage I of germination, and Stage I events result in a fall in the spore core's refractive index and, thus, a loss in optical density at 600 nm due to the large decrease in light scattering by the spore core.[2−4] CaDPA release in Stage I then triggers Stage II, in particular hydrolysis of the spore's peptidoglycan cortex by either of two redundant cortex-lytic enzymes, CwlJ and SleB, in spores of *Bacillus anthracis*, *Bacillus megaterium*, and *Bacillus subtilis*.[2,6,7] Cortex hydrolysis then allows swelling of the spore core and further water uptake, resulting in a core water content similar to that in growing cells and a further fall in the core's refractive index. This full core rehydration completes Stage II of germination and allows metabolism and macromolecular synthesis to begin.[2,4]

Phase contrast microscopy[8] is widely used to examine individual dormant bacterial spores and to characterize their germination.[9−12] In phase contrast microscopy, when the illuminating light passes through specimens, small phase shifts due to differences between the specimen's and the environment's refractive index are converted into amplitude or contrast changes in images, such that dormant spores with their cores' high refractive index appear phase bright and germinated spores appear

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phase dark. Time-lapse phase contrast microscopy has been used to study the kinetics of germination of single *Bacillus* and *Clostridium* spores.[9,10] On the basis of measurements of changes in the intensity of phase contrast images, the germination of single spores has been separated into two distinct stages, an initial Stage I followed by Stage II. As noted above, it has been suggested that Stage I is primarily the release of the spores' CaDPA depot and its replacement by water, and Stage II is largely the degradation of the cortex and expansion and full hydration of the spore core.[9] However, while this suggestion has been generally accepted, it has not been definitively proven.[1−4] Recently, spore germination and outgrowth were analyzed on the basis of phase contrast microscopy, with germination defined as a spore changing from phase bright to phase dark, as measured by a decrease in phase contrast image pixel intensity.[10,12] Time-to-germination (T_{germ}) was then defined as the time at the midpoint of the decreasing pixel intensity, and the distribution of T_{germ} values was measured for a population of *C. botulinum* spores.[12] However, the molecular events before, during, and after T_{germ} are largely unknown, because phase contrast microscopy does not provide direct information on molecular changes.

The kinetics of germination of a spore population is most often monitored by measuring the optical density at 600 nm (OD_{600}) of spore cultures, as this falls ~60% upon completion of Stage II of germination,[2] or by measuring DPA release in bulk-scale with various spectroscopic assays, including UV absorbance spectroscopy,[13] Fourier-transform infrared spectroscopy,[14] and fluorescence spectroscopy measuring DPA release its fluorescence when complexed with Tb^{3+}.[15−17] However, the kinetics of germination of individual spores cannot readily be determined from these population measurements due to significant heterogeneity in the germination of individual spores in populations.[10,12,18]

Micro-Raman spectroscopy and surface-enhanced Raman spectroscopy (SERS) have also been used to monitor the germination of single spores by the analysis of various molecular components including CaDPA, nucleic acids, and proteins.[7,18−21] In combination with optical tweezers and elastic light scattering, Raman spectroscopy can monitor dynamic changes in single cells suspended in liquid and, thus, the heterogeneity in the germination[7,20] and heat inactivation[22,23] of spores in populations. The resolution time of the Raman spectroscopy in the work cited above was 30−60 s and was limited by the low inelastic scattering probability as well as the quantum efficiency of the charge coupled devices (CCD) and spectrographs used.[18,20] Atomic force microcopy has also been used to follow changes in individual spores during germination.[24,25] However, only structural information on the spore surface is obtained by this technique.

Combining phase contrast microscopy and Raman spectroscopy could provide even more information about the germination of single spores, in particular linking changes in spore refractility to specific molecular changes. However, combination of phase contrast microscopy with Raman spectroscopy is not straightforward, since a phase plate is usually placed on the back pupil of phase objectives, and this blocks much of the extremely weak Raman scattering light. In this paper, we present a methodology that combines external phase contrast microscopy, Raman spectroscopy, and optical tweezers to monitor the kinetics of events during the germination of single *Bacillus cereus* spores for up to an hour. The external phase configuration allows the phase plate be placed in an intermediate image plane such that high-throughput objectives can be used for Raman spectroscopy and optical tweezers. In addition, we increased the time resolution of the Raman spectroscopy by approximately an order of magnitude (to ~2 s) using a highly efficient CCD and spectrograph. These changes allowed the rapid recording of phase contrast images and Raman spectra for single germinating spores and, thus, precise correlations between changes in spore refractility and molecular composition.

MATERIALS AND METHODS

Strains Used and Spore Preparation. The *Bacillus* strain used in this work was *Bacillus cereus* T originally obtained from H. O. Halvorson. Spores were prepared at 30 °C in liquid defined sporulation medium, harvested by centrifugation, purified, and stored in water at 4 °C.[26−28] Spores used in this work were free (>98%) of vegetative or sporulating cells, cell debris, and germinated spores, as observed by phase contrast microscopy.

Combination of Phase Contrast Microscopy and Raman Tweezers. An inverted microscope (Nikon, TiS) with an external phase contrast system was equipped with an oil immersion objective (Nikon, Plan Apo 60×, NA = 1.4). The Raman tweezers system is similar the ones used in our previous reports.[18,20] As shown in Figure 1a, the illumination light for phase contrast imaging from a halogen lamp was separated from the backward Raman scattering light with a dichroic mirror (DM). After passing through relay optics and an external phase plate and being filtered by a notch filter to block the near-infrared (NIR) laser, the imaging light arrived at a cooled CCD camera (QSI Inc., 520) to capture the phase contrast image. The collected backward Raman scattering light was separated from the 780 nm excitation laser with another dichroic filter and then focused on the entrance slit of a spectrograph (Princeton Instruments, LS-785). The spectrograph had a resolution of 5 cm^{-1} and was equipped with a back-illuminated deep depletion CCD (Princeton Instruments, PIXIS 400BR) that measures Raman spectra from 600 to 1800 cm^{-1}.

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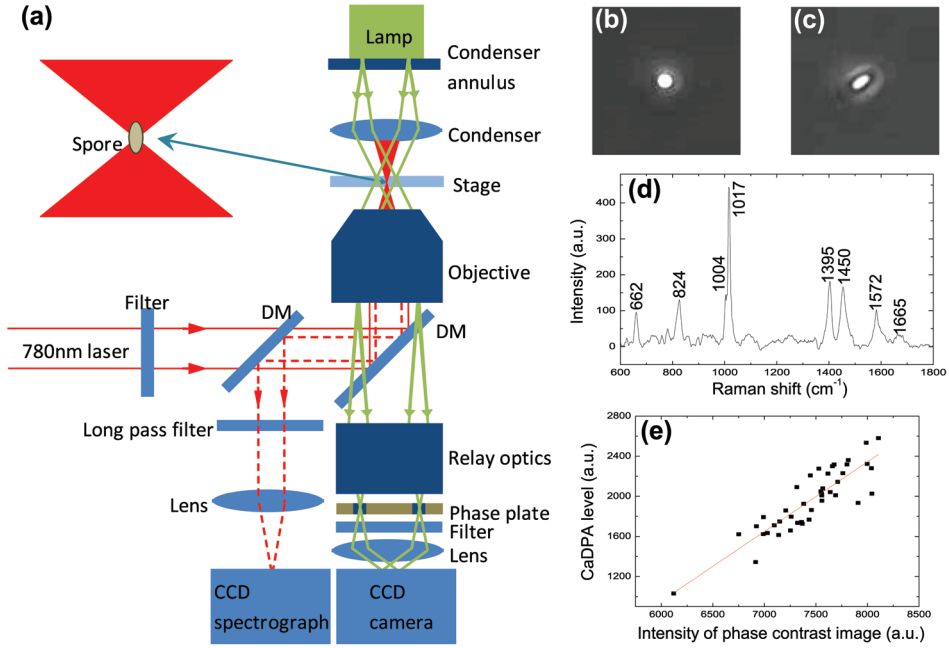

Figure 1. Combined setup for phase contrast microscopy, Raman spectroscopy, and optical trapping. (a) Synthetic schema of the setup. The solid red is the optical trapping and Raman excitation beam, the dashed red is the backward Raman scattering light, and the solid green is the phase contrast imaging light; DM denotes a dichroic mirror. (b) Phase contrast image of a trapped dormant *B. cereus* spore. (c) Phase contrast image of the same spore in picture (b), recorded by temporarily blocking the trapping laser such that the spore realigned. (d) A typical Raman spectrum of a trapped dormant *B. cereus* spore with band intensities expressed in arbitrary units (a.u.). (e) The intensities of the CaDPA-specific band at 1017 cm^{-1} versus the intensities of phase contrast image for 42 trapped single dormant *B. cereus* spores; the red curve is a fitted line.

The high quantum efficiency back-illuminated CCD and high-throughput of the lens-based spectrograph significantly increased the sensitivity of Raman spectroscopy for this instrument; consequently, the time resolution of Raman spectroscopy was increased to 2−10 s.

Monitoring the Germination of Optically Trapped Single Spores. Germinant solutions (300 μL) were first prepared in the microscope sample holder and then heat-activated (30 min at 65 °C and cooled on ice for ≥15 min) spores (5 μL; ~10^6 spores/mL) were added. Time zero of incubation was defined as the time of addition of the spores to the germinant solution. A single spore was trapped at the focus of the NIR laser with a low power of ~3 mW. The phase contrast image of the trapped spore was recorded at a rate of 1 frame/s by the digital CCD camera (1600 × 1200 pixels). Following each exposure, only a small portion of the camera image (21 × 21 pixels, corresponding to an area of 2.5 × 2.5 μm) where the trapped spore was located was recorded, from which the image intensity of the trapped spore was calculated and displayed to monitor the germination in real time. Raman spectra were recorded with a CCD spectrograph. The exposure time for Raman spectroscopy was 2 or 10 s, and the CaDPA level of individual spores was calculated from the intensity of the CaDPA-specific band at 1017 cm^{-1}. The programs for capturing phase contrast images and Raman spectra were controlled synchronously to ensure kinetic correspondence between these two parameters.

Spore Germination on Coverslips. A drop of a suspension of heat-activated spores (20 μL, ~3 × 10^8 spores/mL in water) was placed on the surface of a microscope coverslip. After 1 h at 0 °C to allow spores to adhere, the spore suspension was removed by a pipet, leaving a thin film of spores on the surface of the coverslip. After this thin film was dried in the refrigerator, the slide was washed gently several times to remove unattached spores.

Spores on coverslips were mounted on and sealed to a microscope sample holder at room temperature (23 °C) with ~300 μL of germinant solution on the top of the coverslip. Germinant solutions were 10, 1, or 0.1 mM L-alanine in 25 mM Tris−HCL buffer (pH 7.4) or 30 mM CaDPA produced by a 1:1 mixture of 60 mM $CaCl_2$ and 60 mM DPA that was adjusted to pH 7.5 with Tris base.[20]

Raman Spectroscopy of Single Spores on a Coverslip. A quartz coverslip with large amounts of adhered heat-activated spores was prepared as described above; the germinant solution (300 μL) was added, and the chamber was sealed to prevent evaporation of the liquid in the sample holder. The focus spot of the NIR laser was adjusted to illuminate a single attached dormant spore. A pair of galvanometer optical scanners (Cambridge Technology, 6200H) was used (not shown in Figure 1a) to scan the focused laser beam across an area of 3 × 1.5 μm that covered the selected spore with a scan rate of 2 Hz in the *x*-direction and 20 Hz in the *y*-direction, and Raman spectra were recorded with a 10 s exposure time.

Data Processing of Phase Contrast Images of Large Numbers of Spores. In experiments measuring $T_{release}$ values of heat-activated spores on coverslips, we needed to analyze a large number of spores. Using glass coverslips with adhered spores prepared as described above, the phase contrast image in the full view field (1600 × 1200 pixels) of the CCD camera contained 200−700 spores; three runs of replicate experiments were conducted to accumulate the spore numbers for results

in Figure 5. The full view field of the phase contrast image was recorded at a rate of 1 frame/15 s with the exposure time 0.1 s (~15 s was required to read out and store the full size text images (16 bit, 1600 × 1200 pixels, ~9 MB)). Text images were analyzed by the Matlab program to locate and label individual spores and to calculate spore phase contrast intensities. The $T_{release}$ values for the individual spores were then extracted using Origin to plot the changes in phase contrast image intensities for each spore.

RESULTS

Experimental Setup. The combination of optical tweezers, Raman spectroscopy, and phase contrast microscopy is shown in Figure 1a. A NIR laser at 780 nm served for both optical trapping and the Raman excitation source. An inverted microscope was used with an external phase contrast optical design that allows the microscope to be equipped with a normal objective to obtain phase contrast images. The NIR laser could be focused to form an optical trap by the objective, and more importantly, the Raman scattering light from an optically trapped single spore could also be collected by this objective.[18,20] The phase contrast imaging light was recorded with a digital imaging CCD camera, and the collected backward Raman scattering light was recorded by a high-throughput spectrograph equipped with a back illuminated deep depletion CCD detector. The imaging camera and the CCD spectrograph were controlled by a computer to synchronously capture phase contrast images (Figures 1b and 2b) and Raman spectra (Figures 1d and 2a), respectively. In a typical experiment with a trapped single spore, the time resolution was 1 s for phase contrast images and 2 s or in some cases 10 s for Raman spectra.

CaDPA Level and Phase Contrast Intensity of Dormant Spores. CaDPA comprises ~25% of the spore core dry weight[5,29] and is the most abundant biomarker for *Bacillus* spores. CaDPA-specific Raman scattering bands in dormant *Bacillus cereus* spores are at 662, 824, 1017, 1395, 1450, and 1572 cm^{-1} (Figures 1d and 2a), and the intensities of these bands are directly related to the spores' CaDPA level.[21,26] Figure 1e shows the statistical variation in the CaDPA level (intensity of the 1017 cm^{-1} band) versus the intensities of phase contrast images for 42 trapped individual dormant *B. cereus* spores. The fitted red line indicates that there is a direct correlation between the CaDPA level and the phase contrast intensity of single dormant *B. cereus* spores, with higher CaDPA levels found in spores with higher phase contrast image intensities.

CaDPA Release and Phase Contrast Intensity During Spore Germination. Figure 2a,b shows time-lapse Raman spectra and phase contrast images together with the corresponding 3D intensity profiles of a single trapped *B. cereus* spore germinating with 10 mM L-alanine. The fall in the peak height of CaDPA-specific Raman bands corresponds to CaDPA release. The loss of CaDPA and spore refractility as a function of germination time are shown in Figure 2c,d, respectively, where spore refractility was calculated by normalizing the spore's phase contrast image intensity to its initial value at the first time of measurement (corresponding to that of the dormant spore) after subtraction of

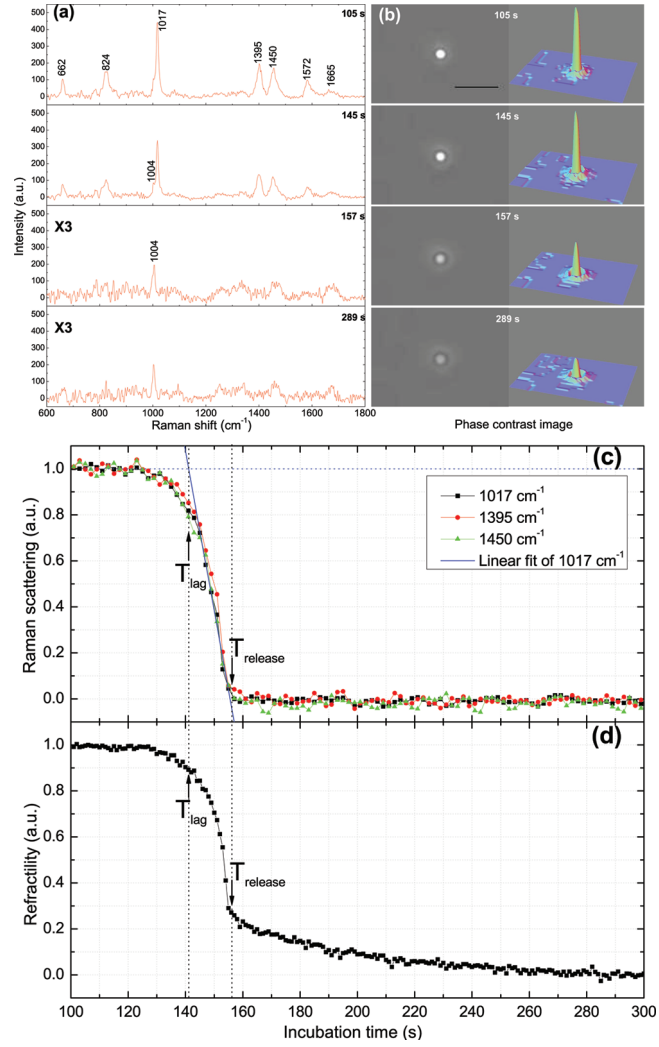

Figure 2. Simultaneous monitoring of L-alanine (10 mM) germination of a single optically trapped *B. cereus* spore by Raman spectroscopy and phase contrast microscopy. (a) Sequential Raman spectra during germination of the single trapped spore. The ×3 in the 157 and 289 s spectra indicate that these spectra were magnified by a factor of 3 for display. (b) Sequential phase contrast images together with their corresponding 3D profile images during germination of the single trapped spore. The scale bar is 5 μm. (c) Intensities of the CaDPA-specific Raman bands at 1017, 1395, and 1450 cm^{-1} as a function of the time in germination of the single trapped spore. The solid blue curve is the fitted line of rapid CaDPA release determined from the intensities of the 1017 cm^{-1} band. (d) Phase contrast image intensities as a function of the time of germination of the single trapped spore as determined by loss of spore refractility. The Raman band intensities and refractility in arbitrary units (a.u.) were normalized to 1 based on values at the first time of measurement. Time resolutions were 2 s for Raman spectroscopy and 1 s for phase contrast images. The upward and downward pointing arrows indicate the T_{lag} and $T_{release}$ times, respectively.

the last unchanged image intensity value (corresponding to that of the fully germinated spore), and Raman band intensities were normalized to the initial values at the first time of measurement. For spore germination with 10 mM L-alanine, CaDPA release was initially slow, albeit significant, and this slow release was followed by a much faster release (Figure 2c). We defined T_{lag} as the time of initiation of rapid CaDPA release and the time for completion of CaDPA release as $T_{release}$ by fitting the CaDPA release data (Figure 2c). Changes in phase contrast image intensity during

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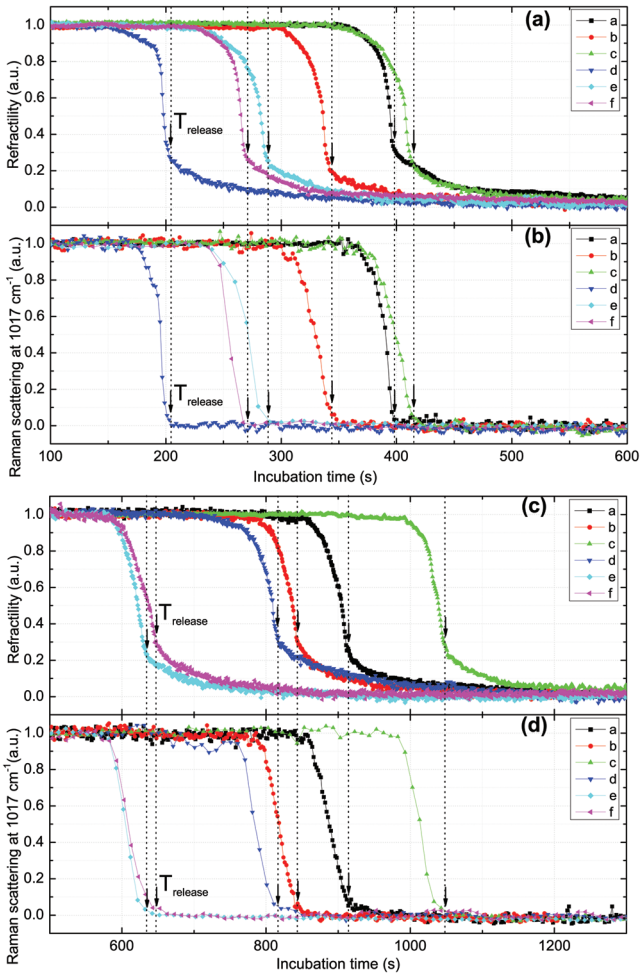

Figure 3. Germination of multiple individual trapped spores monitored by Raman spectroscopy and phase contrast microspcopy. Spores were germinated with 10 mM L-alanine (a, b) or with 30 mM CaDPA (c, d). Germination curves were determined from the loss in spore refractility (a, c) or from the intensities of the CaDPA-specific Raman band at 1017 cm^{-1} (b, d). The refractility and Raman band intensities in arbitrary units (a.u.) were normalized to 1 based on the respective values at the first time of measurement. Downward pointing arrows indicate the $T_{release}$ times for each spore.

L-alanine germination also had three phases (Figure 2d), an initial slow decrease followed by a faster decrease which together resulted in an ~70% decrease in spore refractility and then a second slow decrease to a stable lower refractility. The initial slow decrease in refractility during L-alanine germination of this spore paralleled the initial slow CaDPA release and perhaps also the change in elastic light scattering seen previously just prior to T_{lag};[20] the rapid decrease in spore refractility began at ~T_{lag}, and the end of the rapid loss of refractility was at $T_{release}$ (Figure 2c,d).

Analysis of multiple individual spores germinating with L-alanine gave the same correspondence between CaDPA release and refractility loss (Figure 3a,b; Table 1a). These results indicated that during L-alanine germination there was a slow release of ~13% of CaDPA prior to T_{lag} that was accompanied by an initial slow decrease in spores' phase contrast image intensity of ~10%. These slow changes were then followed by a much more rapid release of essentially all remaining CaDPA paralleled by a rapid decrease in phase contrast image intensity of a further ~64% by $T_{release}$. Following $T_{release}$, the germinating spore's phase contrast image intensity then fell further, reaching a stable value after 2 to 3 min.

In contrast to results with L-alanine germination, during germination with exogenous CaDPA, the spores' rapid release of CaDPA was preceded by at most a minimal period of slow release (Figure 3d), as almost all CaDPA remained in CaDPA germinated spores at T_{lag} (Table 1b). Similar results were obtained when changes in phase contrast image intensities during germination with CaDPA were examined (Figure 3c), as the average phase contrast intensity at T_{lag} was essentially unchanged for CaDPA germinated spores, in contrast to the ~10% decrease during L-alanine germination (Table 1a). Overall, these data strongly suggest that CaDPA release makes the major contribution to the decrease in phase contrast image intensity during spore germination.

In addition to values determined for T_{lag} and $T_{release}$, the period of rapid CaDPA release, ΔT, for *B. cereus* spore germination can also be calculated as $T_{release} - T_{lag}$ (Table 1a,b). For L-alanine germination, the average value of ΔT was ~50% that for CaDPA germination, and for at least L-alanine germination, there was no apparent correlation between values for ΔT and T_{lag}.

It was also notable that the rates of germination of individual spores were very heterogeneous (Figure 3; and see below), as seen previously.[7,9,18,20,26] In particular, while a germination event such as CaDPA release may take up to 60 min for spore populations, for individual spores, the actual time for release of ≥85% of CaDPA during germination is only 1−3 min, although different spores release their CaDPA at very different times after initiation of the germination process.[7,18,20] All reasons for this heterogeneity are not known, but possible reasons include (i) stochastic differences in numbers of nutrient germinant receptors per spore, as the average numbers of receptors per spore are very low; (ii) differences in temperature requirements for heat activation prior to germination between individual spores in populations, perhaps related to differences in core water content between individual spores; and (iii) differences in levels of cortex lytic enzymes in individual spores, in particular CwlJ, whose action in an individual spore is triggered by CaDPA released from that spore in Stage I of germination.[2,4,20,26]

Analysis of Single Spores That Have Not Been Optically Trapped. The single spores analyzed above were all optically trapped, while previous experiments monitoring spore germination by phase contrast imaging used individual spores adhered to microscope slides.[12] Since the microenvironment for surface adhered spores is undoubtedly different from that of spores in an optical trap, it was possible that there might be differences in the germination of spores in these two environments. Consequently, we also analyzed the germination of individual spores adhered to the surface of a microscope coverslip (Figure 4). With the help of the high detection efficiency of our setup, Raman spectra (Figure 4a) could be recorded with a time resolution of ~10 s by scanning the laser spot across the area of a whole single spore adhered to a coverslip (Figure 4b, the spore indicated by the red arrow) with a pair of galvo-mirrors. The results with a single spore on a coverslip germinating with L-alanine again showed that the initial slow decrease in phase contrast image intensity (Figure

Table 1. Values of T_{lag}, $T_{release}$, and ΔT and Percentages of CaDPA and Phase Contrast Intensity (I) Remaining at T_{lag} and $T_{release}$ during the Germination of Individual *B. cereus* Spores[a]

(a)

spore	T_{lag} (s)	$T_{release}$ (s)	ΔT (s)	CaDPA at T_{lag}	CaDPA at $T_{release}$	I at T_{lag}	I at $T_{release}$
a	378	398	20	0.86	0.03	0.89	0.32
b	312	344	32	0.9	0.06	0.92	0.19
c	382	415	33	0.88	0.04	0.90	0.23
d	185	204	19	0.86	0.02	0.89	0.28
e	255	288	33	0.85	0.02	0.88	0.25
f	244	270	26	0.90	0.01	0.92	0.27
average	293±78	320±80	27±6	0.87±0.02	0.03±0.02	0.90±0.02	0.26±0.04

(b)

spore	T_{lag} (s)	$T_{release}$ (s)	ΔT (s)	CaDPA at T_{lag}	CaDPA at $T_{release}$	I at T_{lag}	I at $T_{release}$
a	860	915	55	0.96	0.05	0.97	0.25
b	794	844	50	0.94	0.06	0.95	0.30
c	988	1048	60	0.95	0.02	0.98	0.29
d	762	815	53	0.95	0.04	0.93	0.33
e	582	635	53	0.96	0.02	0.98	0.23
f	582	648	66	0.96	0.04	0.99	0.30
average	761±159	817±158	56±6	0.95±0.01	0.04±0.02	0.97±0.02	0.28±0.04

[a] (a) Values are for optically trapped individual spores germinating with 10 mM L-alanine and have been calculated from the data in Figure 3a,b. (b) Values are for optically trapped individual spores germinating with 30 mM exogenous CaDPA and have been calculated using the data from Figure 3c,d. Values for averages are expressed as averages ± standard deviations.

4c) accompanies slow CaDPA release prior to T_{lag}, this is followed by a rapid loss of most phase contrast image intensity that accompanies rapid and complete CaDPA decrease by $T_{release}$ (Figure 4d), and this in turn is followed by a further fall in phase contrast image intensity to a low stable value in ~2 min. These results are essentially identical to the results with optically trapped single spores during L-alanine germination (Figures 2c,d and 3a,b).

Distribution of $T_{release}$ Values for Individual Germinating Spores. $T_{release}$ is a characteristic time in the germination of individual spores not only when CaDPA release is complete but also when the rapid fall in spore refractility is complete. Given this latter correlation, accurate values of $T_{release}$ for individual spores can then be obtained from analysis of phase contrast images alone, even though there is a further slow decrease of ~30% in phase contrast intensity after $T_{release}$. To demonstrate the power of the latter analysis for studying the germination heterogeneity of individual spores, we used phase contrast microscopy to analyze the germination of large numbers of individual spores adhered on a glass coverslip to determine their $T_{release}$ values, since $T_{release}$ values are known to be heterogeneous for individuals in spore populations.[18,20] In previous work examining the heterogeneity in germination by following individual spores by phase contrast microscopy,[12] the time to germination was defined as the midpoint of the curve given by the decrease in phase contrast intensity. However, $T_{release}$ as observed in current work is a more precise time point, as observed by both phase contrast microscopy and Raman spectroscopy. Figure 5a gives the probability distribution of $T_{release}$ values for ~500 spores germinating with 10 mM L-alanine, and with this saturating germinant concentration, $T_{release}$ values were essentially all within the initial 100−500 s. In contrast, subsaturating 0.1 mM L-alanine gave significantly slower germination (Inset, Figure 5a), and the distribution of $T_{release}$ values was shifted to longer times, with significant numbers of spores exhibiting $T_{release}$ values up to ~2500 s (Figure 5b). The probability distribution of $T_{release}$ times for spores germinating with 30 mM CaDPA indicated that most spores had completed CaDPA release within 1000−1400 s, although a few required >4000 s (Figure 5c).

DISCUSSION

Phase contrast microscopy has been used for many years to characterize the germination of bacterial spores.[9] Although the change in spore refractility from phase bright to phase dark during germination has long been considered to be directly related to the release of the spore's large CaDPA depot, until now, there has been no direct experimental demonstration that both CaDPA release and changes in the phase contrast image intensity are simultaneous in single spores. However, in the current work, a combination of phase contrast microscopy, Raman spectroscopy, and optical tweezers as well as improvement in the time resolution of the Raman spectroscopy has been used to show that during the germination of individual spores both CaDPA release and decrease in phase contrast image intensity are simultaneous. Indeed, ~70% of the decrease in spore phase contrast image intensity during germination was simultaneous with CaDPA release.

The results obtained with this new technology have also led to a number of new conclusions about *B. cereus* spores and their germination. One is that the intensity of the phase contrast image of an individual dormant *B. cereus* spore is directly proportional to the level of the spore's CaDPA. Presumably, spores with higher CaDPA levels also have lower core water levels and, thus, elevated solid/water ratios in their core. One intriguing possibility is that these latter spores may well have higher moist heat resistance compared to the spore population, since lower core water levels almost always are correlated with increased spore moist heat resistance.[5] This heterogeneity in spore levels of CaDPA may be one of the reasons for the heterogeneity in levels of moist heat resistance between individual spores in populations.[26] Conse-

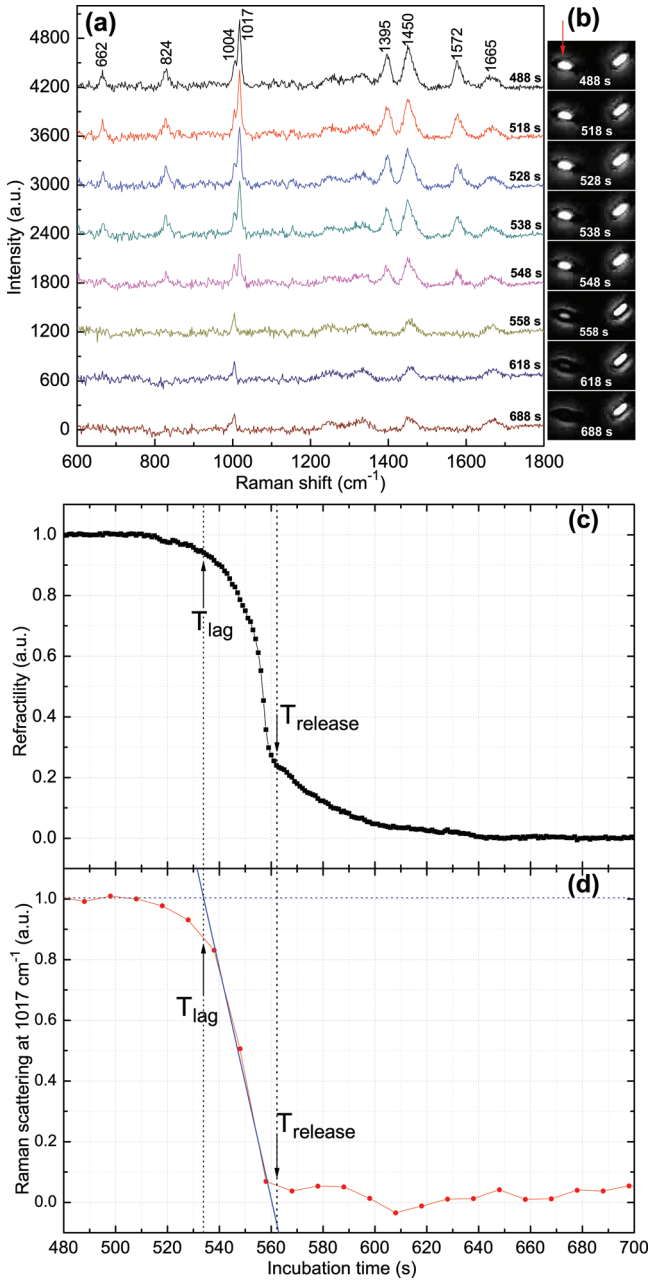

Figure 4. Germination of a single spore on a coverslip with 1 mM L-alanine monitored by Raman spectroscopy and phase contrast microspcopy. Raman spectra (a) and phase contrast images (b) were taken at various times in germination of the single spore. In (b) the spore indicated by the red arrow is the one measured. (c, d) Germination of the single spore as assessed by losses of spore refractility (c) or the intensity of the CaDPA-specific Raman band at 1017 cm^{-1} (d). The solid blue curve in (d) is the fitted line of rapid CaDPA release determined from the intensities of the 1017 cm^{-1} band. The refractility and Raman band intensities in arbitrary units (a.u.) were normalized to 1 based on the respective values at the first time of measurement. The time resolution for phase contrast images was 1 s and for Raman spectroscopy was 10 s. The upward and downward pointing arrows in (c) and (d) indicate the T_{lag} and $T_{release}$ times, respectively.

quently, the relationship between a spore's phase contrast image intensity and its CaDPA level observed in this work could have potential applications in the determination of CaDPA levels using phase contrast microscopy alone, and this in turn may be useful for analysis of the heterogeneity of spore moist heat resistance

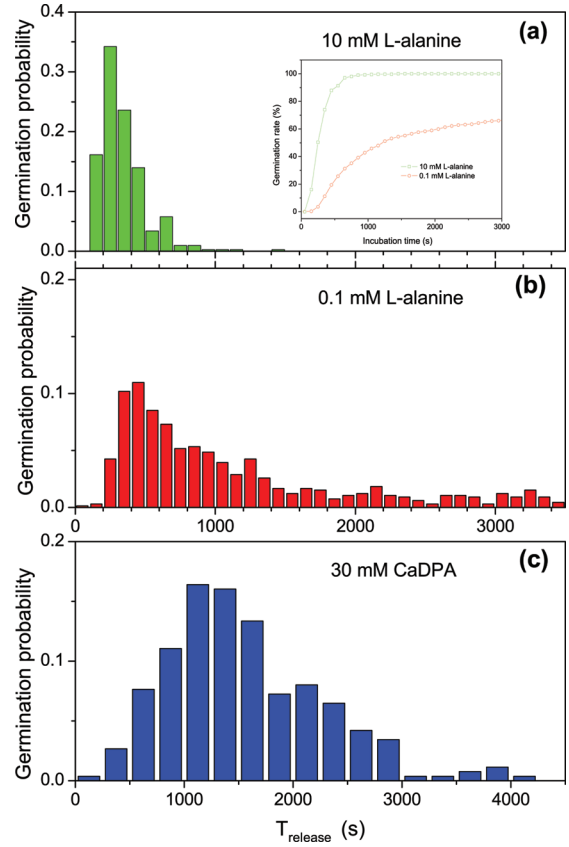

Figure 5. Probability distribution of $T_{release}$ times during germination of large numbers of individual spores determined by phase contrast microscopy. $T_{release}$ times during observation of ~500 (a), ~900 (b), or ~500 (c) spores adhered on a coverslip and incubated with 10 mM (a) or 0.1 mM (b) L-alanine or 30 mM CaDPA (c) were determined by phase contrast microscopy. 99%, 75%, and 93% of the observed spores germinated in 90 min in (a), (b), and (c), respectively. Germination probability is the percentage of the number of spores with a $T_{release}$ value in a particular 100 s (a, b) or 250 s (c) interval over the total number of germinated spores in 90 min.

as well as other spore properties. Indeed, analysis of the germination of ~500 individual *B. cereus* spores with 10 mM L-alanine by phase contrast microscopy alone found no correlation between the spore's initial phase contrast image intensity (and thus CaDPA level) and its $T_{release}$ value (data not shown).

A second conclusion from this work is that, during germination of single *B. cereus* spores in L-alanine or CaDPA, the end of the rapid drop in spore refractility revealed by phase contrast microscopy precisely corresponds to the time of completion of CaDPA release $T_{release}$ as revealed by Raman spectroscopy. Previously, the germination of single spores was separated into an initial Stage I and a subsequent Stage II, based mainly on the change of spore refractility, suggesting that Stage I is primarily the release of the spore's CaDPA depot and its replacement by water, and Stage II is largely the complete degradation of the cortex and the expansion and full hydration of the spore core.[9] The present work found that $T_{release}$ is the demarcation point between Stages I and II. The first two changes in spore refractility during L-alanine germination paralleled CaDPA release, suggesting that CaDPA release alone is responsible for these two periods of change in spore refractility. However, it is possible that some small amount of

cortex hydrolysis possibly catalyzed by CwlJ may facilitate CaDPA release during germination.[20] Elucidation of roles for cortex hydrolysis in decreases in spore refractility during germination will, thus, require analysis of CaDPA release and spore refractility during the germination of spores lacking one or both of the two redundantly essential cortex-lytic enzymes, CwlJ and SleB. Such mutants are available for spores of *B. subtilis* and *B.* megaterium[2,7] but not *B. cereus*.

A third conclusion is that during Stage II, when the spore refractility decreased by ~30% in 2 and 3 min and after the spore's CaDPA had been completely released, there were additional minor changes in the spores' Raman spectra (Figure 2a). With further improvements in the sensitivity of Raman spectroscopy, it might be possible in the future to elucidate some of the molecular changes in Stage II of germination using this type of technology.

A fourth conclusion is that the rapid CaDPA release during L-alanine germination was preceded by a much slower rate of CaDPA release, although only the fast release was seen for germination by exogenous CaDPA. While the reasons for this difference in the germination with these two germinants is not yet known, this observation suggests a likely scenario in nutrient germination of a single spore as follows: (1) L-alanine activates a spore's germinant receptors; (2) this activation triggers slow CaDPA release; (3) slow CaDPA release triggers the action of the spore's cortex-lytic enzyme CwlJ that is activated by CaDPA from either exogenous or endogenous sources;[2] and (4) a small amount of CwlJ action results in a large increase in the rate of CaDPA release. This scenario is consistent not only with the data in the current work but also with previous work that indicates that CaDPA release from *cwlJ B. megaterium* and *B. subtilis* spores is much slower than from the corresponding *sleB* or wild-type spores.[20] In contrast, exogenous CaDPA causes spore germination by activating CwlJ, thus causing only rapid CaDPA release and not the initial slow CaDPA release. While the scenario above is by no means proven, it seems likely and could be proven by analysis of CaDPA release and changes in refractility during germination of spores lacking one or both redundant cortex-lytic enzymes.

A fifth conclusion is that the correspondence between the initial slow decreases and the ensuing rapid decreases in both refractility and CaDPA seen with spores germinating in solution in an optical trap were also seen with spores germinating when adhered on a coverslip. This similarity in results between these two methods strongly indicates that neither optical trapping nor surface adhesion have significant effects on changes in either CaDPA release or phase contrast image intensity during spore germination. This further suggests that phase contrast microscopy alone could be used to extract germination parameters such as $T_{release}$ from experiments with individual spores. To demonstrate this, we studied spore germination heterogeneity by simultaneously monitoring the germination of hundreds of individual spores using phase contrast microscopy and extracting the $T_{release}$ values for individual spores. Analysis of the distribution of these $T_{release}$ values revealed that: (i) with a lower concentration of L-alanine, the spores have longer and extended $T_{release}$ values compared to a saturating level of L-alanine; and (ii) with CaDPA as the germinant, the distribution of $T_{release}$ values is shifted to much longer times than in germination by L-alanine, even at low L-alanine concentrations.

In summary, the methodologies presented here provide major advances in the ability to analyze spore germination, in particular in the analysis of the germination of large numbers of individual spores. It seems certain that application of these methodologies will provide new important insight into the process of bacterial spore germination. The combination of phase contrast microscopy and laser tweezers Raman spectroscopy is applicable not only to the study of spore germination but also to studies of other single microorganisms suspended in liquid.

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