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Monitoring the Kinetics of Uptake of a Nucleic Acid Dye during the Germination of Single Spores of *Bacillus* Species

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Dormant bacterial spores do not take up and bind nucleic acid dyes in the spore core but readily take up such dyes when they are fully germinated. We present a methodology that combines fluorescence microscopy, phase contrast microscopy, and laser tweezers Raman spectroscopy to monitor the kinetics of uptake of the nucleic acid dye SYTO 16 during germination of individual *Bacillus cereus* and *Bacillus subtilis* spores. The level of dye bound to nucleic acids of individual spores was measured by fluorescence emission, while changes in spore refractility and the level of the 1:1 chelate of dipicolinic acid and Ca^{2+} (CaDPA) were monitored by phase contrast microscopy and Raman spectroscopy, respectively. The results obtained include (1) during nutrient germination, SYTO 16 began to enter the spore core and bind to nucleic acids just when spores had released all CaDPA and continued until hydrolysis of spores' peptidoglycan cortex was complete; (2) during germination with exogenous CaDPA, rapid SYTO 16 uptake began only 2−7 min after complete release of endogenous CaDPA for both *B. cereus* and *B. subtilis* spores; (3) the rate but not the timing of dye uptake and the maximum level of dye bound to nucleic acid were increased during nutrient germination of *B. subtilis* spores lacking ~75% of the DNA binding proteins that normally saturate dormant spore DNA; (4) SYTO 16-DNA binding was not observed during nutrient germination of *B. subtilis* spores lacking the protease that degrades spores' DNA binding proteins, even after cortex hydrolysis; (5) SYTO 16 uptake by germinating *B. subtilis* spores lacking the cortex-lytic enzyme (CLE) CwlJ was low, again even after cortex hydrolysis, although SYTO 16 uptake by germinating spores lacking the other redundant CLE SleB was even higher than in germinating wild-type spores; and (6) there was no SYTO 16 uptake by germinating spores that lacked both CwlJ and SleB, even after CaDPA release. These results suggest that during spore germination SYTO 16 uptake is minimal until CaDPA has been released and DNA binding proteins have been degraded and further that CLEs' degradation of the spore cortex plays a crucial role in uptake of this dye.

Dormant bacterial spores of *Bacillus* species are very resistant to harsh conditions.[1] One factor in spore resistance is the low permeability of the dormant spores' inner membrane surrounding the central core, the site of spore nucleic acids.[1−5] As a consequence of the low inner membrane permeability, many toxic small molecules as well as nucleic acid dyes cannot enter the dormant spore core.[2−6] While spores can remain dormant for years, exposure to a number of specific chemicals rapidly triggers spores' return to life in the process of germination.[7−10] Spore germination takes place in two stages. In stage I, nutrient germinants bind to specific receptors located in the spore's inner membrane, triggering the release of core small molecules, most notably the large depot (~25% of core dry wt) of dipicolinic acid (DPA) present in a 1:1 chelate with divalent cations, predominately Ca^{2+} (dipicolinic acid and Ca^{2+} (CaDPA)); the small molecules released are replaced by water. In stage II, the peptidoglycan cortex is hydrolyzed, and in spores of *Bacillus* species, cortex hydrolysis is catalyzed by either of two redundant cortex-lytic enzymes (CLEs), CwlJ and SleB; cortex hydrolysis then allows core expansion and further water uptake followed by spore outgrowth.[7−10] However, there is no information on precisely how and when external small molecules cross the inner spore membrane and enter the spore core during germination.

SYTO 16 is a membrane-permeant nucleic acid dye that exhibits a large fluorescence enhancement upon binding to nucleic

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Table 1. Values of $T_{release}$, $\Delta T_{release}$ ($T_{release} - T_{lag}$), ΔT_F ($T_F - T_{release}$), ΔT_{maxF} ($T_{maxF} - T_{release}$), and I_{maxF} (Maximum Fluorescence Intensity) during Germination of Spores of *B. cereus* and Various *B. subtilis* Strains[a]

spores (germination or genotype)	$T_{release}$ (min)[1]	$\Delta T_{release}$ (min)	ΔT_F (min)	ΔT_{maxF}(min)	I_{maxF} (fluo. unit) (I_{maxF}/I_{maxF_PS533})
B. cereus (L-alanine)	4.1 ± 1.4	0.7 ± 0.2	3.2 ± 0.6	8.5 ± 1.5	83.6 ± 9.5 (0.69 ± 0.08)
B. cereus (CaDPA)	13 ± 4.6	0.9 ± 0.2	8.2 ± 1.7	15.9 ± 3.4	165.6 ± 34.2 (1.38 ± 0.28)
PS533 (wild-type, L-alanine)	15.3 ± 8.5	2.5 ± 0.6	7.6 ± 0.9	12.9 ± 4.8	120.1 ± 20.5 (1.00 ± 0.17)
PS533 (wild-type, CaDPA)	53.1 ± 17.8	7.1 ± 2.9	26.1 ± 6.5	40.2 ± 10.2	256.8 ± 78.1 (2.14 ± 0.65)
PS578 ($\alpha^-\beta^-$, L-alanine)	9.1 ± 4.6	2.7 ± 0.8	3.7 ± 0.9	6.4 ± 1.1	232.7 ± 39.3 (1.94 ± 0.33)
PS1029 (*gpr*, L-alanine)	10.3 ± 4.23	1.83 ± 0.5	NA	NA	NA
FB111 (*cwlJ*, L-alanine)	34.8 ± 10.8	19.3 ± 9.1	22.4 ± 6.3	35.8 ± 7.2	19.2 ± 4.5 (0.16 ± 0.04)
FB112 (*sleB*, L-alanine)	15 ± 7.5	1.7 ± 0.4	13.3 ± 3.8	21.9 ± 3.8	136.9 ± 21.2 (1.14 ± 0.18)
FB113 (*cwlJ sleB*, L-alanine)	40.8 ± 14.5	24.8 ± 4.8	NA	NA	NA

[a] Values were determined and normalized as described in the legends to Figures 3, 4 and S1 (Supporting Information) and are averages and standard deviations for results with 30 individual spores germinating when adhered to a coverslip. I_{maxF}/I_{maxF_PS533} is the value of the maximum SYTO 16 fluorescence intensity reached for spores of each species/strain compared to that for wild-type *B. subtilis* spores (strain PS533) germinating with L-alanine; the value for the latter spores was set at 1.00.

acids.[2−5] SYTO 16 cannot penetrate the dormant spore core.[2,3] However, when spores are fully germinated, SYTO 16 is able to cross the spores' inner membrane, bind nucleic acids, and exhibit strong green fluorescence.[2−5] Indeed, SYTO 16 has been used to distinguish dormant and germinated spores by flow cytometry.[2,4] However, it is unclear how and when this dye gets into germinating spores, in particular for single spores germinated by nutrient or non-nutrient germinants, although it appears likely that most dye uptake will require hydrolysis of the spore cortex.[2,4,9]

In previous work, fluorescence in germinated spore populations due to SYTO 16 binding was detected by fluorescence microscopy[3] and flow cytometry.[2,4,5] However, these methods alone are unable to link SYTO 16 uptake during germination to events such as CaDPA release and cortex hydrolysis, in particular for individual spores. Recently, Raman spectroscopy and optical tweezers have been combined with phase contrast microscopy, differential interference contrast microscopy, and elastic light scattering to characterize the kinetics of germination of individual spores.[10−12] These methods have revealed a precise correspondence between a rapid drop in spore refractility and the release of the great majority of spore's CaDPA, followed by a slower further drop of ~30% in spore refractility due to cortex hydrolysis. However, the kinetics of uptake of nucleic acid dyes and their binding to nucleic acids during germination of individual *Bacillus* spores has not been examined. There has been a report using a nucleic stain to look at the kinetics of germination of spore populations,[13] but the intrinsic heterogeneity in the germination of individual spores in populations could not be observed by such a technique. Coote et al. reported the use of confocal scanning laser microscopy (CSLM) to simultaneously measure the loss of refractility using phase contrast microscopy and changes in permeability to ethidium bromide using fluorescence microscopy with individual germinating *Bacillus cereus* spores.[14] This work showed there was a large increase in ethidium bromide permeability during spore germination. However, the resolution time (3−7 min) obtained in this work was significantly longer than the time needed for the rapid drop in the refractility of *B. cereus* spores (~1 min, see Table 1), and this precluded precise delineation of kinetic processes during these spores' germination. In addition, this method was unable to link ethidium bromide uptake to specific molecular events such as CaDPA release by individual spores during their germination.

In this work, we have used Raman tweezers in combination with fluorescence and phase contrast microscopy to observe the kinetics of uptake of SYTO 16 during the germination of individual *B. cereus* and *B. subtilis* spores. Raman spectroscopy was used to monitor CaDPA release; phase contrast microscopy was used to monitor the whole germination process via changes in spore refractility, and fluorescence microscopy was used to determine the level of SYTO 16 that had crossed the spore's inner membrane and bound to nucleic acid in the spore core. With this new method, we have increased the time resolution for Raman spectroscopy and fluorescence and phase contrast imaging to 5−10 s per frame such that the dynamic germination of individual spores can be monitored with excellent time resolution.

MATERIALS AND METHODS

Bacterial strains used, spore preparation and germination, experimental methods, and data processing can be found in the Supporting Information.

RESULTS

Experimental Setup. The experimental setup for the combination of fluorescence microscopy, phase contrast microscopy, and Raman spectroscopy of single germinating spores is shown in Figure 1a. A collimated light-emitting diode (LED) served as the fluorescence excitation source and the phase contrast illumination light. A near infrared (NIR) laser at 780 nm served for both optical trapping and the Raman excitation source. An inverted microscope with an external phase contrast optical design was used to observe the phase contrast image.[10] Two charge coupled device (CCD) cameras (Fluo. CCD and PC CCD) and one CCD spectrograph were used to record the fluorescence (Fluo.) images, phase contrast (PC) images, and Raman spectra, respectively. As an example, Figure 1b,c and Figure 1d,e show phase contrast and the fluorescence images, respectively, of wild-type *B. subtilis* spores adhered on a coverslip and germinated with 10 mM L-alanine. Prior to germination, the dormant spores appeared phase bright (Figure 1b) and no SYTO 16 fluorescence was observed (Figure 1d). Twenty minutes later, many of the

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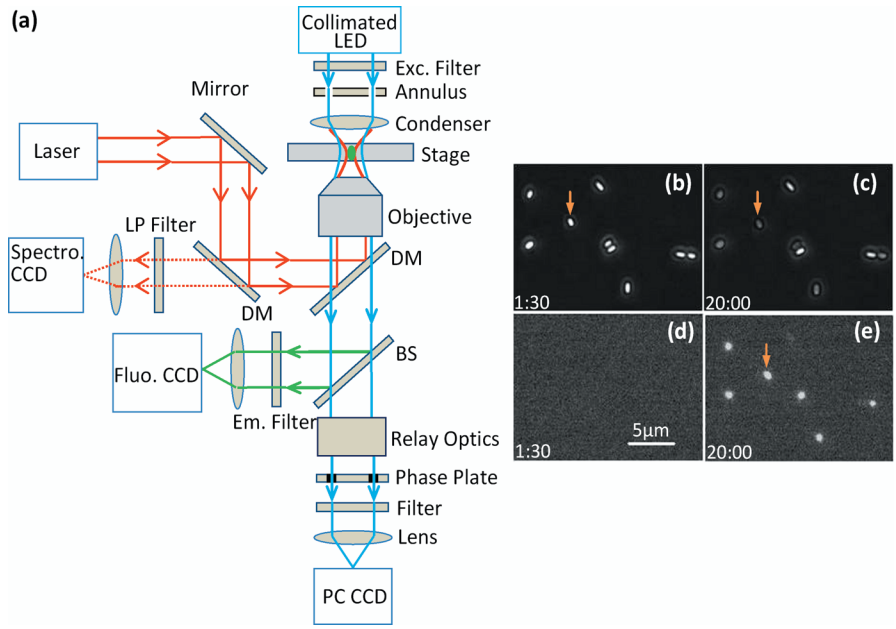

Figure 1. (a) Synthetic scheme of the combined setup for fluorescence microscopy, phase contrast microscopy, and Raman tweezers. The blue line is the fluorescence excitation and phase contrast illumination LED light; the green line is the fluorescence from germinated spores collected by the objective. The solid red line is the optical trapping and Raman excitation light, and the dotted red line is the backward Raman scattering light collected by the objective: DM, dichroic mirror; BS, beam splitter prism; Exc. Filter, excitation filter at 455 nm; Em. Filter, emission filter at 530 nm; LP Filter, long pass filter; Fluo. CCD, CCD camera for fluorescence imaging; PC CCD, CCD camera for phase contrast imaging; and Spectro. CCD, Spectrograph CCD for Raman spectroscopy. (b, c) and (d, e) are the phase contrast images and fluorescence images, respectively, of PS533 (wild-type) *B. subtilis* spores attached on a coverslip and germinated with L-alanine for 90 s (b, d) or 20 min (c, e). The red arrow indicates the same spore that at 90 s was still dormant, as it appeared phase bright (b), exhibited no fluorescence (d) and, after 20 min, had fully germinated, as it was phase dark (c) and exhibited strong SYTO 16 fluorescence (e).

spores had fully germinated and appeared phase dark (Figure 1c), and these germinated spores exhibited significant SYTO 16 fluorescence (Figure 1e).

Fluorescence Images, CaDPA Release, and Phase Contrast Intensities during Germination of Individual *B. cereus* Spores. Figure 2a−c shows time-lapse Raman spectra, phase contrast images, and fluorescence images, respectively, from a single optically trapped *B. cereus* spore germinating with 1 mM L-alanine. In Figure 2a, the fall in the peak heights of CaDPA-specific Raman bands at 662, 824, 1017, 1395, 1450, and 1572 cm^{-1} correspond to CaDPA release during spore germination. The release of CaDPA (intensity of the 1017 cm^{-1} Raman band), loss of refractility, and change in the fluorescence of the spore as a function of germination time are shown in Figure 2d. The times indicated by the arrows in Figure 2d were defined as follows: (i) T_{lag}, the time of initiation of rapid CaDPA release; (ii) $T_{release}$, the time for completion of rapid CaDPA release; (iii) T_F, the time at the end of the rapid increase in SYTO 16 fluorescence; and (iv) T_{maxF}, the time at which the SYTO 16 fluorescence reached its maximum value. The curves in Figure 2d show the following results: (i) germinating spores exhibited a minimal increase in SYTO 16 fluorescence prior to $T_{release}$; (ii) SYTO 16 fluorescence began to increase rapidly at $\sim T_{release}$ and along with the second slower phase of loss in spore refractility due to the degradation of the spore cortex with attendant spore core swelling and water uptake;[10,12] and (iii) following the rapid initial increase in SYTO 16 fluorescence, the fluorescence increased slowly, eventually reaching its maximum value at T_{maxF}. Analysis of multiple individual *B. cereus* spores adhered on a coverslip and germinated with 1 mM L-alanine (Figure 3a) gave similar results. The large heterogeneity in the timing of T_{lag} during the germination of individual heat-activated spores in Figure 3a is likely determined by different levels of germination receptors (GR) per spore and the stochastic binding/triggering of GRs by L-alanine.[10−12]

While the nutrient germination analyzed above proceeds via spores' nutrient germinant receptors, spores can also germinate with exogenous CaDPA.[7,8,15] The spore's extremely high level of intracellular CaDPA was measured from the Raman spectrum of the spore, and the relatively small contribution of the extracellular CaDPA to this Raman spectrum was subtracted.[12] CaDPA germination does not proceed via the nutrient germinant receptors but rather by activation of the CLE CwlJ.[9,15] Figure 3b shows the changes in refractility and fluorescence for multiple individual *B. cereus* spores adhered on a coverslip and germinating with 30 mM CaDPA in the presence of SYTO 16. Compared with L-alanine germination (Figure 3a), CaDPA germination exhibited the following different features: (i) SYTO 16 fluorescence did not begin its rapid increase at $T_{release}$ but only after a 2 to 3 min delay during which there was at most a very slow fluorescence increase; (ii) the period of rapid fluorescence increase lasted ~8 min, much longer than the ~3 min for germination with L-alanine (Table 1); and (iii) the maximum intensity of the SYTO 16 fluorescence was higher than that with L-alanine germination (Table 1). Similar features were also observed during CaDPA germination of *B. subtilis* spores (Table 1; and see below).

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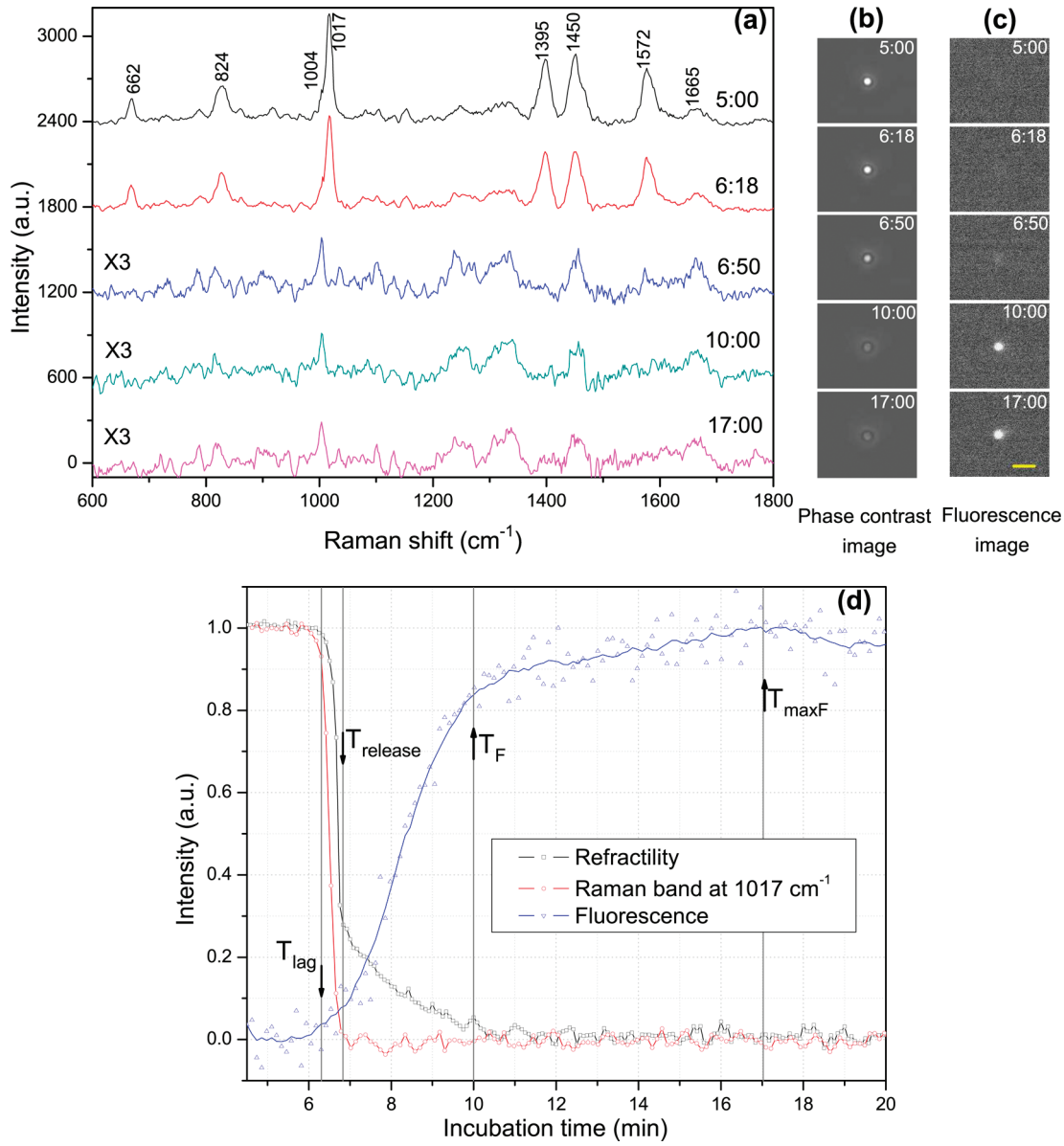

Figure 2. Simultaneous recording of fluorescence images, phase contrast images, and Raman spectra of a single optically trapped *B. cereus* spore germinating with L-alanine. (a–c) Sequential Raman spectra, phase contrast, and fluorescence images, respectively, during spore germination. The ×3 in (a) indicates that these spectra were magnified by a factor of 3 for display. The scale bar in (c) is 3 μm. (d) Intensities of the fluorescence, the CaDPA specific Raman band at 1017 cm^{-1}, and refractility as a function of germination time; the solid gray lines indicate the times of T_{lag}, $T_{release}$, T_F, and T_{maxF}. Spore refractility was calculated by normalizing a 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 the last unchanged image intensity value (corresponding to that of the fully germinated spore). Raman band intensities were normalized to the initial values at the first time of measurement, and the fluorescence intensities were normalized to the maximum intensity value. All intensities are given in arbitrary units (a.u.).

Analysis of SYTO 16 Uptake during Germination of Individual *B. subtilis* Spores of Various Strains. We also germinated wild-type, $\alpha^-\beta^-$, and *gpr B. subtilis* spores with 10 mM L-alanine and again followed the uptake and binding of SYTO 16 to nucleic acid. Figure 4a–c shows the changes in refractility and fluorescence for multiple individual wild-type, $\alpha^-\beta^-$, and *gpr* spores, respectively, adhered on a coverslip. The results of this analysis indicated that (i) spores of all three strains exhibited very similar losses of CaDPA and overall refractility, indicating that all of these spores germinated normally, consistent with published results;[16,17] (ii) the times for the SYTO 16 fluorescence increase with $\alpha^-\beta^-$ spores (times $\Delta T_F = T_F - T_{release} \approx 4$ min and $\Delta T_{maxF} = T_{maxF} - T_{release} \approx 6$ min) were much faster than those for wild-type spores ($\Delta T_F \approx 8$ min and $\Delta T_{maxF} \approx 13$ min); (iii) the maximum SYTO 16 fluorescence intensity reached by the germinating $\alpha^-\beta^-$ spores was higher than with wild-type spores; and (iv) the SYTO 16 fluorescence with *gpr* spores was much lower than with wild-type spores, even well after full CaDPA release and cortex hydrolysis. These results suggest that (1) the SYTO 16 fluorescence of germinated spores is due the binding of this dye to DNA in the spore core; and (2) saturation of spore DNA by α/β-type SASP prevents SYTO 16 binding to DNA (see Discussion).

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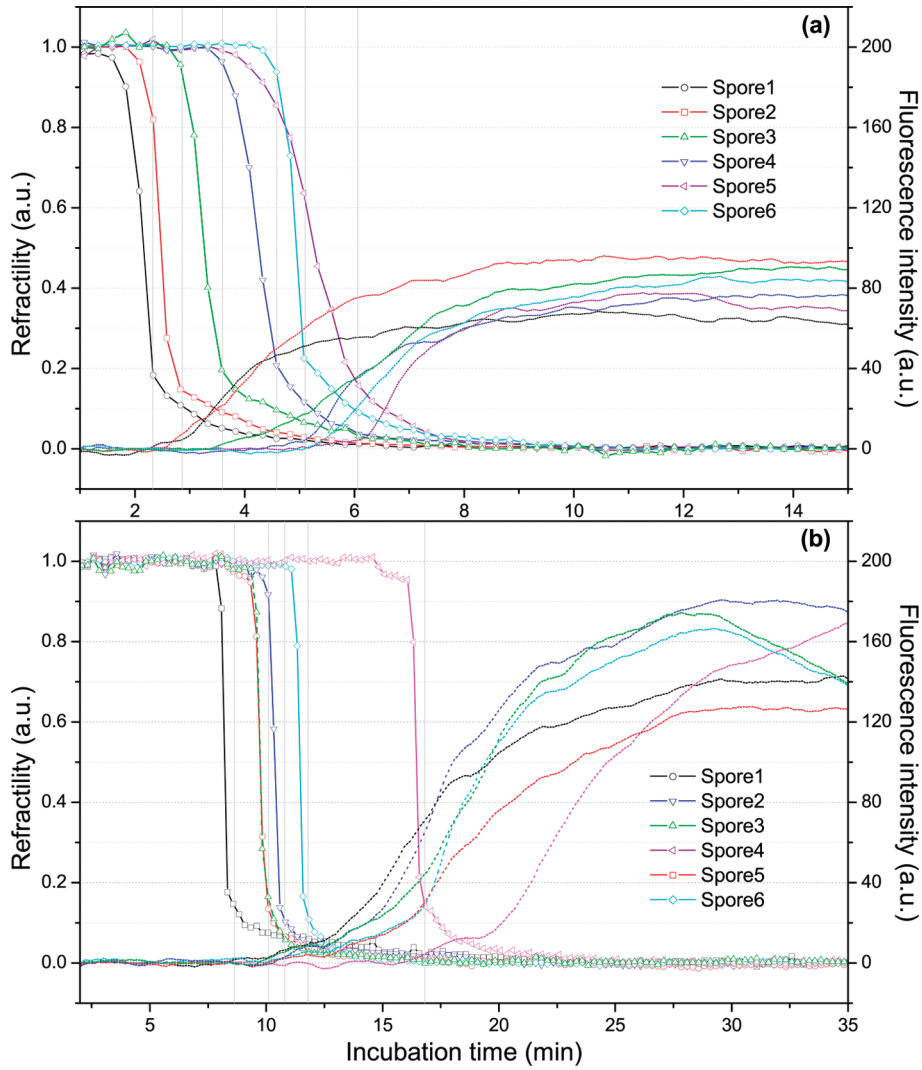

Figure 3. Germination of multiple individual *B. cereus* spores adhered on a coverslip determined by changes in refractility and SYTO 16 fluorescence. Spores were germinated with either L-alanine (a) or CaDPA (b). The solid gray lines in the figure indicate the $T_{release}$ times for each spore. The fluorescence intensities in arbitrary units (a.u.) were normalized to the fixed fluorescence CCD count intensity under the experimental situation of 4 mW/cm^2 of LED excitation light. The same normalization parameters were used in both (a) and (b) as well as in Figure 4 and Figure S1 (Supporting Information).

We also germinated wild-type *B. subtilis* spores with exogenous CaDPA in the presence of SYTO 16 (Figure 4d). The CaDPA germination of these spores exhibited similar features to the CaDPA germination of *B. cereus* spores, and these were different from those of L-alanine germination (compare Figures 3a and 4a and 3b and 4d). In particular for *B. subtilis* spores germinating with CaDPA: (i) there was a delay of ~4−7 min before SYTO 16 fluorescence began to increase rapidly; (ii) it took much longer for the fluorescence increase to be complete than with L-alanine germination; and (iii) the maximum fluorescence intensity reached was higher than with L-alanine germination (Table 1).

As noted above, it appears likely that cortex hydrolysis has major effects on SYTO 16 uptake during spore germination. In order to investigate this likelihood in detail, we followed the L-alanine germination of *B. subtilis* spores lacking either or both of the two CLEs, CwlJ and SleB, that play essential but redundant roles in spore germination.[3] Figure S1a−d (Supporting Information) shows the release of CaDPA (intensity of the 1017 cm^{-1} Raman band) and changes in refractility and SYTO 16 fluorescence of optically trapped single spores lacking either CwlJ (Figure S1a, Supporting Information) or SleB (Figure S1c, Supporting Information), and the changes in refractility and fluorescence intensities of multiple individual *cwlJ* (Figure S1b, Supporting Information) or *sleB* (Figure S1d, Supporting Information) spores adhered on a coverslip and germinating with L-alanine, respectively. The results from these experiments (Supporting Information, Figure S1; Table 1) indicated that (i) during *cwlJ* spore germination, CaDPA release corresponding to the initial phase of spore refractility loss was much slower than in wild-type spores, as seen previously;[9,12] (ii) while SYTO 16 fluorescence began to appear just after full CaDPA release with *cwlJ* spores, the time to reach maximum fluorescence was much greater than with wild-type spores and the maximum SYTO 16 fluorescence intensities reached were lower; (iii) for germinating *sleB* spores, CaDPA release and the loss of refractility were the same as for wild-type spores, although the maximal SYTO 16 fluorescence in the germinated *sleB* spores was somewhat higher than with wild-type spores, but both ΔT_F and ΔT_{maxF} were increased by a factor of ~2; and (iv) with germinating spores that lacked both CwlJ and SleB, the loss of refractility was almost the same as with

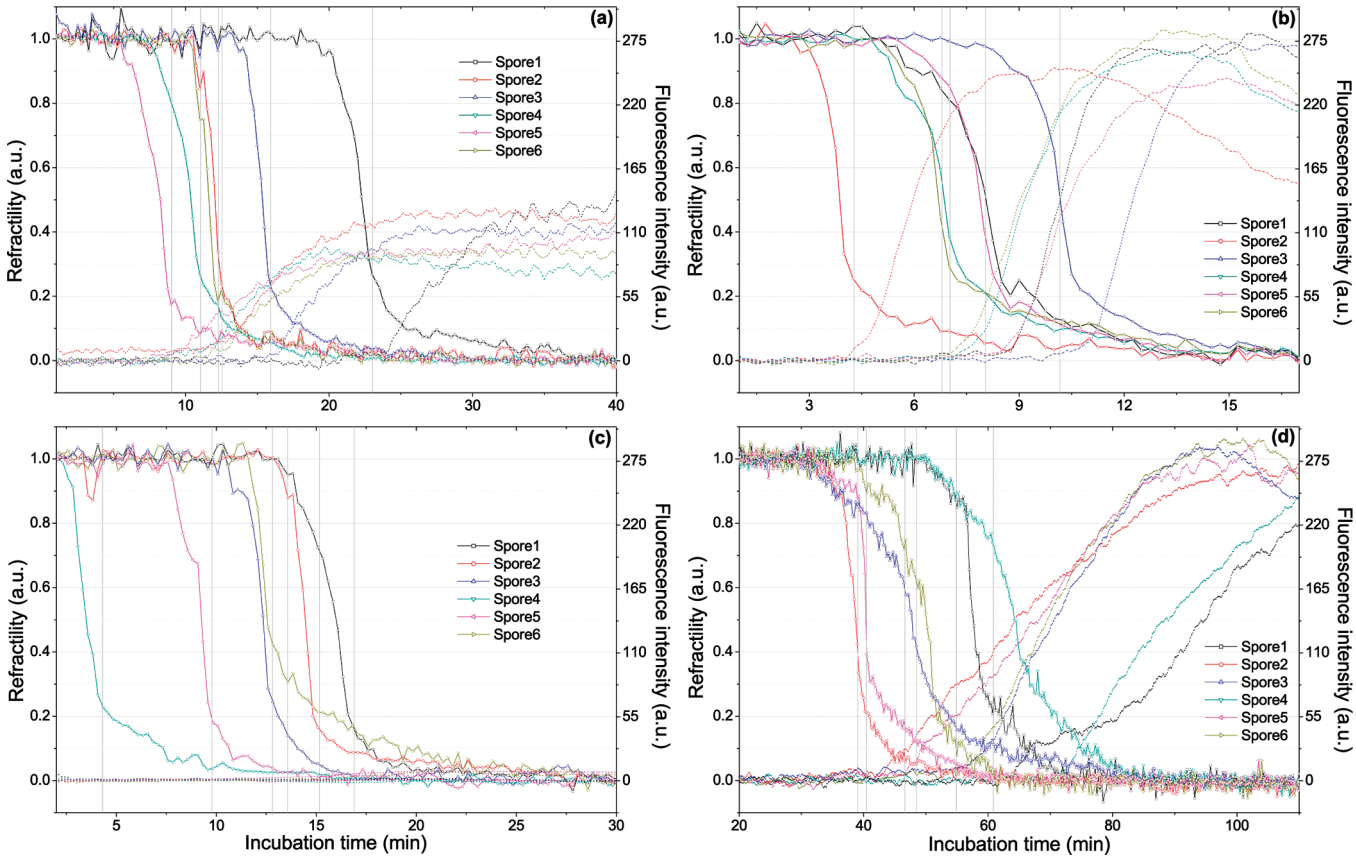

Figure 4. Changes in refractility and SYTO 16 fluorescence during germination of multiple *B. subtilis* spores adhered on a coverslip. The *B. subtilis* spores used were from strains: (a, d) PS533 (wild-type), (b) PS578 ($\alpha^-\beta^-$), and (c) PS1029 (*gpr*). (a–c) L-alanine germination, (d) CaDPA germination. Normalization of refractility and fluorescence intensities were as described in the legends to Figures 2 and 3.

cwlJ spores as was the release of CaDPA (data not shown), as seen previously;[9,12] however, no SYTO 16 fluorescence was observed with germinating *cwlJ sleB* spores, even long after CaDPA release (Table 1; and data not shown).

DISCUSSION

A combination of fluorescence microscopy, phase contrast microscopy, and Raman tweezers has been achieved in the current work. This new technology can simultaneously record fluorescence images, phase contrast images, and Raman spectra of either single or multiple individual bacterial spores, allowing detailed characterization of the spore germination process. Establishing the relationship between SYTO 16 binding to DNA by observing the spores' fluorescence signal along with other changes associated with spore germination, including loss of refractility by phase contrast microscopy and changes in molecular composition by Raman spectroscopy, has further allowed a number of conclusions about the spore germination process, some of which are novel ones.

The first conclusion is that prior to T_{lag} in germination, neither *B. cereus* nor *B. subtilis* spores accumulated little if any SYTO 16, as found previously for dormant spores,[2−4,6] and there was at most only a minimal amount of SYTO 16 uptake during the $\Delta T_{release}$ period. This was the case even if the spores lacked the great majority of their DNA-binding α/β-type SASP that saturate spore DNA and at least in vitro block binding of nucleic acid dyes to DNA [Setlow, P. unpublished results]. Presumably, during these time periods, the spore's inner membrane's permeability is still too low to allow passage of SYTO 16 into the spore core. This further indicates that the process of commitment, whereby a spore continues on in germination even if a nutrient germinant is removed, does not significantly alter spores' inner membrane permeability, since commitment precedes CaDPA release by several min for individual *B. subtilis* spores germinating at 37 °C.[18]

A second conclusion is that the great majority of the fluorescence due to SYTO 16 uptake into germinating spores is due to binding to DNA. This dye can bind to both DNA and RNA, and while SYTO 16 fluorescence when bound to RNA is lower than when bound to DNA, spores contain 3- to 4-fold more RNA than DNA.[19] Consequently, one would expect that binding to RNA would generate the most fluorescence upon SYTO 16 uptake by spores. However, (i) there was no SYTO 16 fluorescence from germinating *gpr* spores through at least 30 min, even though CaDPA release and spore refractility loss were normal; and (ii) there is no degradation of SASP-α and -β for at least 60 min after initiation of germination of *B. subtilis gpr* spores.[17] These two findings suggest that the presence of α/β-type SASP on DNA prevents SYTO 16 binding just as the saturation of DNA with α/β-type SASP in vitro prevents binding of SYTO dyes to DNA [P. Setlow, unpublished results]. In addition, (i) α/β-type SASP do not bind to RNA;[1,20,21] and (ii) during germination of $\alpha^-\beta^-$ spores, the rate of SYTO 16 fluorescence increase was significantly

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faster and the maximum fluorescence achieved was significantly higher than with wild-type spores. These latter two observations further suggest that binding to RNA contributes very little to the SYTO 16 fluorescence seen in germinating wild-type *B. subtilis* spores. Presumably the tRNA and rRNA in germinating spores are largely bound to proteins, and this greatly decreases the binding of SYTO 16 to these RNAs, just as α/β-type SASP binding greatly decreases SYTO 16 binding to DNA. There will also only be minimal mRNA levels in germinating spores until well after completion of full cortex hydrolysis when rapid spore macromolecular synthesis begins.[17,22]

The third conclusion is that there are three notable differences in the kinetics and intensity of SYTO 16 fluorescence during L-alanine and CaDPA germination of both *B. cereus* and *B. subtilis* spores. (1) During L-alanine germination, SYTO 16 fluorescence increases rapidly beginning at $T_{release}$ while during CaDPA germination there is a 2−7 min delay after $T_{release}$ before SYTO 16 fluorescence begins to increase. (2) The rate of SYTO 16 fluorescence increase during CaDPA germination is slower than for L-alanine germination, even when the greater fluorescence intensity of CaDPA germinated spores is taken into account. (3) The maximum SYTO 16 fluorescence intensity of CaDPA-germinated spores is ∼2-fold higher than that of L-alanine-germinated spores. The reasons for these differences in the timing, rate, and amount of SYTO 16 uptake during CaDPA germination are not clear. However, CaDPA germination does not proceed through the spores' germinant receptors but rather by the activation of CwlJ, perhaps directly, by the exogenous CaDPA.[15] Presumably, the process of cortex hydrolysis when exogenous CaDPA activates CwlJ is different in some way than when CaDPA released from a spore's core activates CwlJ. However, what these differences in patterns of cortex hydrolysis might be and how any such differences would affect SYTO 16 uptake and binding to spore DNA are not clear.

A fourth conclusion, and certainly one giving new insight into spore germination, is that there must be significant α/β-type SASP degradation during the process of cortex hydrolysis, and thus, SASP degradation does not begin only after cortex hydrolysis is complete. This is indicated by the elimination of SYTO 16 binding to spore DNA during *gpr* spore germination, even after cortex hydrolysis was complete. Since α/β-type SASP block SYTO 16 binding to DNA in germinating spores and SYTO 16 binds significantly to spore DNA during the period of cortex hydrolysis in wild-type spore germination, there must be significant α/β-type SASP degradation during this latter period even though the core's water content has almost certainly not yet reached that of a fully germinated spore. As a consequence, and not perhaps surprisingly, the demarcation between stage II of spore germination and the initiation of spore outgrowth events such as SASP degradation is not exact.

The fifth conclusion is that CLE action is crucial for SYTO 16 uptake, since spores lacking both CwlJ and SleB exhibited no SYTO 16 fluorescence during germination, even though these spores released all CaDPA. During germination of spores that contained only CwlJ (*sleB* spores), times to reach T_F and T_{maxF} were also significantly longer than with wild-type spores, suggesting that cortex hydrolysis is slower when only CwlJ is available for cortex hydrolysis, and when only SleB was available (*cwlJ* spores), times to reach T_F and T_{maxF} during spore germination were even longer than with *sleB* spores. Why having only one CLE gives slower cortex hydrolysis during spore germination is not clear, although there are a number of potential contributing factors including the following: (1) There is simply less CLE activity with only one of the two CLEs present. (2) CwlJ and SleB are present in different locations in spores, with CwlJ on the outer edge of the cortex, and SleB at both the inner and outer edges.[23−25] Thus, CwlJ hydrolysis might proceed from the outside of the cortex inward and give different kinetics of cortex hydrolysis during germination than would SleB action, that can presumably take place from both the inner and outer surfaces of the cortex. However, even if such a difference in the pattern of cortex hydrolysis by these two CLEs exists, how such a difference might affect SYTO 16 uptake is not clear. (3) The mechanisms of activation of CwlJ and SleB during spore germination are clearly quite different, CwlJ being activated by CaDPA and SleB in some other fashion, perhaps by water uptake following or concomitant with release of endogenous CaDPA.[7,15] (4) The specificity of CwlJ and SleB for cortex cleavage is also different, as SleB is a lytic glycoslase and CwlJ has some other, as yet undetermined, specificity.[26] This plus the fact that the structure of the cortical peptidoglycan differs going from the inner edge of the cortex to the outer edge[27] suggests that action of these different CLEs might have different dynamic effects on overall cortex structure during spore germination. However, again, how differences in the rates and overall pattern of cortex hydrolysis during germination might affect spores' inner membrane permeability and, thus, SYTO 16 uptake is not clear. It is also notable that the maximum SYTO 16 fluorescence intensity seen during L-alanine germination was much lower with *cwlJ* spores than with wild-type spores. However, the reason for this difference is not clear. It is also striking that SYTO 16 incorporation into wild-type spores began so early after initiation of cortex hydrolysis, e.g., almost immediately upon completion of CaDPA release even when core expansion was presumably minimal. This suggests that there must be significant changes in inner membrane permeability when only small amounts of cortex peptidoglycan have been degraded. However, how cortex peptidoglycan degradation causes a change in inner spore membrane permeability is not known and is an important topic for future work.

In summary, the combination of fluorescence microscopy, phase contrast microscopy, and Raman tweezers have enabled us to investigate the germination of large numbers of individual spores by simultaneously following three characteristic changes in spore germination: DNA binding by SYTO 16, loss of refractility, and CaDPA release. Detailed analysis of the relationships between these three events has allowed a number of notable conclusions

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about the spore germination process. In addition, the new microscopy technology developed in this work should also be applicable to studies of other single microorganisms.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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