Characterization of single heat-activated *Bacillus* spores using laser tweezers Raman spectroscopy

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Abstract: Heat activation of dormant bacterial spores is a short treatment at a sublethal temperature that potentiates and synchronizes spore germination. In this paper, laser tweezers Raman spectroscopy (LTRS) was used to study the heat activation of single spores of *Bacillus cereus* and *Bacillus subtilis*. We measured the Raman spectra of single spores without treatment, during heat activation at 65 °C (*B. cereus*) or 70 °C (*B. subtilis*), and following heat activation and cooling to 25 °C. Principle component analysis (PCA) was applied to discriminate among the three groups of spores based on their Raman spectra. The results indicated that: (1) there are large changes in the Raman bands of Ca-DPA and protein for both *B. cereus* and *B. subtilis* spores during heat activation, indicative of changes in spore core state and partial protein denaturation at the heat activation temperatures; (2) these spectral changes become smaller once the heated spores are cooled, consistent with heat activation being reversible; (3) minor spectral differences between untreated and heat-activated and cooled spores can be discriminated by PCA based on non-polarized and polarized Raman spectra; and (4) analysis based on polarized Raman spectra reveals that partial denaturation of protein during heat activation is mainly observed in the vertically polarized component.

OCIS codes: (170.5660) Raman spectroscopy; (170.1530) Cell analysis

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1. Introduction

Bacterial spores of *Bacillus* species formed upon starvation are metabolically dormant, extremely resistant to heat, desiccation, UV irradiation and chemicals, and can survive for long periods without nutrients [1]. Dormant spores can also germinate and outgrow into vegetative cells when the environment is favorable [2], although significant portions of dormant spore populations often fail to germinate [3]. However, heat activation by treatment of spores at a sublethal temperature for a short time (e.g. 30 min at 65 °C for *Bacillus cereus* and 70 °C for *Bacillus subtilis*) significantly increases the percentage of spores that germinate [4–7]. Under natural conditions, the activation of dormant spores in water occurs slowly, but a

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heat activation step most often potentiates germination of >90% of spore populations [4]. Spore heat activation is also reversible, as heat activated spores can slowly revert to their original dormant state when stored at lower temperatures [4,7]. However it is not clear if this reversal is complete, and whether there are any irreversible changes in spore components accompanying heat activation [4]. When dormant spores of several *Bacillus* species are exposed to increasing temperatures, they undergo an endothermic transition around 55°C, as shown by calorimetry studies [8–10]. It has been suggested that this change is the result of a transition of the spore core from a glass-like state in dormant spores to a rubbery-like state for activated spores [10], although this has by no means been proven [8].

The exact mechanism of spore heat activation is still unclear, but may involve reversible breakage of bonds such as hydrogen bonds in some spore proteins. It was suggested that partial denaturation of proteins may be associated with the breaking of disulfide bonds during activation [11], and that this is reversed by reoxidation [4]. Heat activation also causes a loss of some spore coat protein and slight changes in the ultrastructure of the spore coat, as well as some release of the 1:1 chelate of Ca^{2+} and pyridine-2,6-dicarboxylic acid (dipicolinic acid (DPA)) (Ca-DPA) that comprises ~25% of the dry wt of the spore's central region or core [1,2,7,12]. Recently, heat activation was also found to cause some release of Ca-DPA and protein from a spore population [12]. However, it is not clear that the Ca-DPA and protein released came from the majority of spores, or only from a small percentage of spores that are especially sensitive to the sublethal temperatures used for heat activation, and thus completely release their Ca-DPA and are killed. To definitively assess of the fate of Ca-DPA and protein during spore heat activation, analysis of the composition of individual spores before, during, and after heat activation might be most useful.

Raman spectroscopy has been widely used for single cell studies [13] and in biomedical applications [14], due to its high sensitivity and rapid response to subtle structural changes in molecules in cells. Combined with confocal microscopy and optical trapping, Raman spectroscopy (known as laser tweezers Raman spectroscopy (LTRS)) has been used for non-destructive, non-invasive analysis of single cells suspended in liquid [15–18], in particular for the analysis of the germination of single *Bacillus* spores [19,20]. LTRS allows individual dormant spores suspended in liquid to be captured by a focused near-infrared laser beam and characterized by Raman spectroscopy, from which information about the biochemical composition and environment of individual spores can be obtained. Recently, LTRS has also been used for identification and sorting of single microbial cells [21, 22], analysis of the levels of Ca-DPA in single dormant spores [16] and bacterial lysis [23]. In addition to normal Raman spectroscopy, polarized Raman spectroscopy is also a useful technique for studying molecular structure and the depolarization ratio of Raman bands provides important information about the symmetry of a normal vibration [24–27]. Polarized Raman spectroscopy has been successfully applied to detection of early dental caries [25], studying the glass transition of polymers [26] and determining the orientation of double-stranded RNA [27].

In this work, we have applied LTRS for studying heat activation of single spores of *B. cereus* and *B. subtilis*. By measuring Raman spectra of single spores before, during and after heat activation, we hoped to determine if there are differences in molecular composition and structure between untreated and heat-activated spores, and how the spores' spectra change during heat activation. To determine if individual untreated spores, heat-activated spores, and spores during heat activation can be differentiated by their Raman spectra, we applied principle component analysis (PCA) [22]. Since heat activation is reversible, changes in Ca-DPA and proteins of heat-activated spores could be subtle. Therefore we developed a novel polarized Raman spectroscopy technique combined with laser tweezers, which allows simultaneous measurement of both the parallel and vertical polarization components of the Raman scattering light from single trapped spores illuminated with a linearly polarized excitation beam. Here, we demonstrate that minor differences between untreated and heat-

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activated spores can be discriminated by multivariate PCA based on non-polarized and polarized Raman spectra, and further that the partial denaturation of spore proteins during heat activation is reflected mainly in the vertically polarized component.

2. Experimental details

2.1 Polarized LTRS

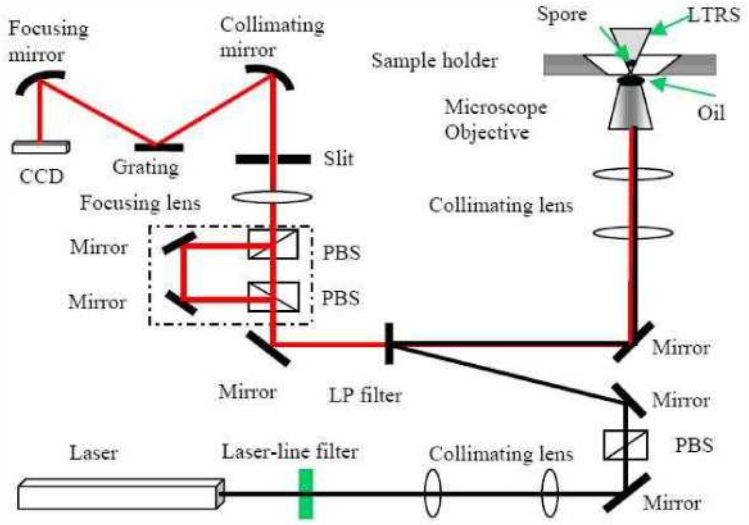

Fig. 1. Optical layout of polarized laser tweezers Raman spectroscopy

The optical layout of the LTRS system used in our work is depicted schematically in Fig. 1. The light source for trapping spores and exciting Raman spectra was from a near-infrared diode laser (TIGER, Sacher Lasetechnik) at 795nm, and frequency components above 795nm were blocked with a laser-line filter. The laser beam passed through a PBS polarizer so that the trapping and excitation beam was linearly polarized in the horizontal direction. The linearly polarized beam was reflected by a Raman edge long-wave pass filter (Semrock Corp., LP01-808U-25) and then introduced into an inverted microscope (Nikon, TE2000-S) to form a laser tweezers. The microscope was equipped with a 100x objective with NA=1.30. Spore samples suspended in distilled water were loaded into a sample holder, and a single spore was trapped in the waist of the light beam. The same laser was used to excite the trapped spore, and the backscattering Raman light was collected by the same objective and returned in the same optical path. However, the edge long-pass filter allowed the Raman scattering light to pass through, and blocked stray scattering light. To record the conventional (non-polarized) Raman spectra, the transmitted Raman scattering light was focused onto the entrance slit of a spectrograph (Jobin Yvon, Triax 320) equipped with a nitrogen-cooled charge-coupled detector (CCD). Inside the spectrograph, the Raman light was dispersed by a reflection grating and imaged by the CCD. Raman spectra were recorded from 400 to 1900 cm^{-1} with a spectral resolution of ~ 6 cm^{-1}.

The Raman scattering light from the trapped spores contains two polarization components, parallel and perpendicular to the horizontal polarization direction of the incident beam. To simultaneously record the parallel and perpendicular (vertical) polarized spectra, a pair of polarization beam splitters (PBS) and a pair of reflection mirrors (highlighted by the dashed rectangular box in Fig. 1) were inserted into the optical path to separate the parallel and vertical (with respect to the polarization of incident light) components in the vertical dimension. The two components were then imaged onto different rows on the CCD chip separated by a few tens of pixels so that they could be acquired simultaneously.

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2.2 Heat-activation of bacterial spores

The *Bacillus* species used in this work were *B. cereus* T and *B. subtilis* PS832, a prototrophic derivative of strain 168. Spores of both species were prepared, purified and stored at 4 °C as described [28–30]. All spore preparations were free (>98%) of growing or sporulating cells and germinated spores as determined by phase contrast microscopy. Heat activation caused an ~5-fold increase in the percentage of *B. cereus* spores that germinated with the nutrient germinant inosine, and an ~3-fold increase in the *B. subtilis* spores' germination with L-valine, and in both cases ≥95% of the heat-activated spores germinated with these germinants (data not shown). Each spore sample was diluted with distilled water to ~10^7 spores/ml and transferred to three tubes. Tube 1 was kept at 25 °C during measurements, and contains untreated spores. Tube 2 was immersed for 30 min in a water bath at 65 °C (*B. cereus*) or 70 °C (*B. subtilis*) and then cooled on ice for 15 min, a standard procedure for heat activation of *Bacillus* spores. Subsequently, the cooled spores were loaded onto a sample holder kept at 25 °C, and the Raman measurements on individual spores were made. The spores in tube 3 were first treated at 65 °C (*B. cereus*) or 70 °C (*B. subtilis*) for 30 min, and then loaded into the sample holder kept at the same temperatures. The sample holder on the microscope was temperature-stabilized with a thermoelectric controller (Thorlabs Inc., TEC2000).

2.3 Raman spectra acquisition of individual spores

For each group of samples, the Raman spectra of up to 50 individual spores were collected with a laser power of 30 mW and an acquisition time of 40 s. To reduce the influence of stray scattering light, a background spectrum without a spore in the trap was obtained under the same conditions, and was subtracted from the spectra of individual spores. Polystyrene beads with diameters of 2μm were also loaded into the sample holder, and the beads' spectra were recorded for Raman intensity calibration.

2.4 Principal component analysis (PCA)

The algorithm of PCA used in this work was described previously for the discrimination of different bacterial species [22]. Following spectral acquisition, the spectra of individual untreated spores, cooled heat-activated spores, and heat-activated spores held at the heat-activation temperature were pre-processed. The background spectrum was subtracted from each spectrum and three-point average smoothing was applied. The first derivative of each Raman spectrum was taken to eliminate the baseline effect in the discrimination. The derivative spectra were normalized to the area and the range was reduced to highlight the fingerprint region (600 to 1800 cm^{-1}) for PCA analysis.

The PCA algorithm used in the analysis was provided in the multivariate statistical analysis toolbox PLS-toolbox (Eigenvector Research Inc., Manson, Wash.). For PCA, the original spectra are expressed as a linear combination of a set of orthogonal components (referred as principle components or loading spectra), and the coefficients are called scores [24]. The first three PCA coefficients (scores) were projected on orthogonal axes as a first classifier of identification for untreated spores, cooled heat-activated spores, and spores held at heat-activation temperature. 25 spectra of individual spores from each group were randomly chosen as the training sets to establish the loading spectra and 5 spectra of individual spores were randomly chosen as the testing sets. The testing sets were projected on the three-dimension space built by the first three PC scores of training sets to confirm the correctness of the spore identification [22, 24].

3. Experimental Results and Discussion

3.1 Average Raman spectra

Figure 2 shows the averaged non-polarized Raman spectra of *B. cereus* and *B. subtilis* spores before and after heat activation, as well as during heat activation at an elevated temperature.

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The positions of main characteristic bands are marked. The peaks at 659, 824, 1017, 1395, 1446 and 1572 cm^{-1} are due to the vibration modes of Ca-DPA [16, 19]. The bands at 1004 and 1604 cm^{-1} are assigned to phenylalanine in spore proteins. The bands at 1655, 1667, and 1671 cm^{-1} are associated with the α-helical, nonregular, and β-sheet structures of proteins' amide I (peptide bond C==O stretch), respectively [31]. The difference spectra d and e show the differences between untreated spores and cooled heat-activated spores or spores held at the heat activation temperature, respectively. Tables 1 and 2 give the intensity ratios of major Raman bands for *B. cereus* and *B. subtilis* spores, respectively.

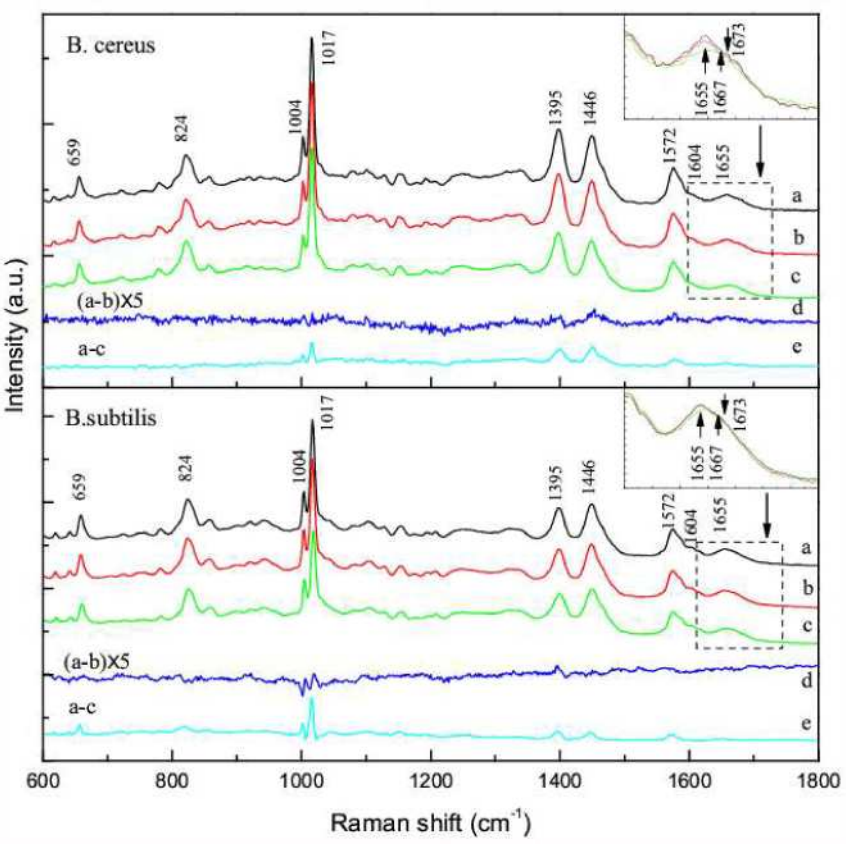

Fig. 2. Non-polarized Raman spectra of *B. cereus* and *B. subtilis* spores. Curve a is for untreated spores at 25 °C; curve b for cooled heat-activated spores measured at 25 °C; curve c for spores during heat-activation measured at the elevated temperature (65 °C for *B. cereus*, and 70 °C for *B. subtilis*); curve d is the subtraction between curves a and b, magnified by a factor 5 for display, and curve e is the subtraction between curves a and c. The insets show the magnified spectra in the range of the protein amide I bands. The spectra were averaged over 50 spores and the vertical baselines were shifted for display.

Table 1. Ratios of the intensities of characteristic Raman bands of *B. cereus* spores

	I_{1017}/I_{824}	I_{1395}/I_{824}	I_{1446}/I_{824}	I_{1572}/I_{824}
BC at 25 °C	3.03±0.09	1.79±0.06	1.56±0.07	1.24±0.048
BC at 65 °C	2.61±0.08	1.33±0.06	1.09±0.03	1.01±0.04
BC heated/cooled	3.09±0.17	1.79±0.12	1.53±0.06	1.22±0.06

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Table 2. Ratios of intensities of characteristic Raman bands of *B. subtlis* spores

	I_{1017}/I_{824}	I_{1395}/I_{824}	I_{1446}/I_{824}	I_{1572}/I_{824}
BS at 25 °C	2.19±0.08	0.97±0.05	1.06±0.04	0.87±0.05
BS at 70 °C	1.92±0.08	0.85±0.04	0.98±0.04	0.81±0.03
BS heated/cooled	2.15±0.06	0.92±0.04	1.03±0.03	0.83±0.02

The results shown in Fig. 2 indicate that when the spores were held at heat activation temperatures, there was a large decrease in the band intensities of Ca-DPA and phenylalanine. In addition, there were large changes in the intensity ratios among the Ca-DPA bands; the intensity ratios of bands at 1017, 1395, 1446 and 1572 cm^{-1} to that of the band at 824 cm^{-1} were significantly decreased for spores of both species, suggesting that the environment of Ca-DPA inside the spore core was changed at the elevated temperature (Tables 1 and 2). However, the decrease in the Ca-DPA band intensities does not imply there has been a significant decrease in total spore Ca-DPA during heat activation, since when the heat-activated spores were cooled to 25 °C, both the intensities and intensity ratios of the Ca-DPA bands returned to close to values for untreated spores (curves d in Fig. 2; and Tables 1 and 2). The other finding (insets, Fig. 2) was that the intensity of the amide α-helical band at 1655 cm^{-1} was slightly decreased, and a shoulder at 1667 cm^{-1} (amide nonregular structure) appeared at the heat-activation temperature, indicative of a protein conformational change upon thermal denaturation. It has been reported that the transition from an α-helix to a disordered structure upon heating can sometimes be partially reversed upon cooling [32]. However, once spores are killed by heat, the frequency shift of proteins' Raman bands is irreversible [33].

In our experiments, once the heat-activated spores were cooled, the difference spectra between the heat-activated and untreated spores became very small (curve d in Fig. 2), and the intensity ratios of the cooled heat-activated spores were close to those of the untreated spores (Tables 1 and 2). These results indicate that many of the effects of heat-activation treatment are reversible, in agreement with previous studies [4, 32]. The data in Table 1 and 2 also show that the variation between spores in the same group was much less than the differences between spores of different groups. For example, for the untreated *B. cereus* spores at 25 °C, the ratio of I_{1017}/I_{1014}=3.03±0.09 and for the heat-activated *B. cereus* spores at 65 °C I_{1017}/I_{1014}=2.61±0.08. However, after the spores were cooled, this value became 3.09±0.17. Similar behavior was observed for *B. subtilis* spores, and together these data indicate that the changes observed upon heat activation were due to similar changes in the great majority of the individual spores.

3.2 PCA results

A PCA algorithm was used to determine if minor differences between single untreated spores, cooled heat-activated spores and spores during heat activation can be differentiated based on their individual Raman spectra. The PCA results are shown in Fig. 3. The score plots in Figs. 3(a) and 3(b) show that the spores of *B. cereus* and *B. subtilis* held at heat-activation temperatures can be completely separated from the spores in the other two groups, mainly by the scores in the second principal component (PC2). However, the PCA scores of the untreated and cooled heat-activated spores appeared to overlap (Figs. 3(a) and 3(b)). These results were in agreement with the analysis based on the average spectral intensities (Section 3.1): there was a large change in non-polarized Raman spectra of the spores when they were held at the heat-activation temperature and this change became small when the heat activated spores were cooled. The significance of Figs. 3(a) and 3(b) is that these findings were valid for almost all individual spores of both *B.cereus* and *B. subtilis*, while the result in Fig. 2 was

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the average over a population of spores. Again, these data are consistent with minimal if any, loss of Ca-DPA from individual spores upon heat activation.

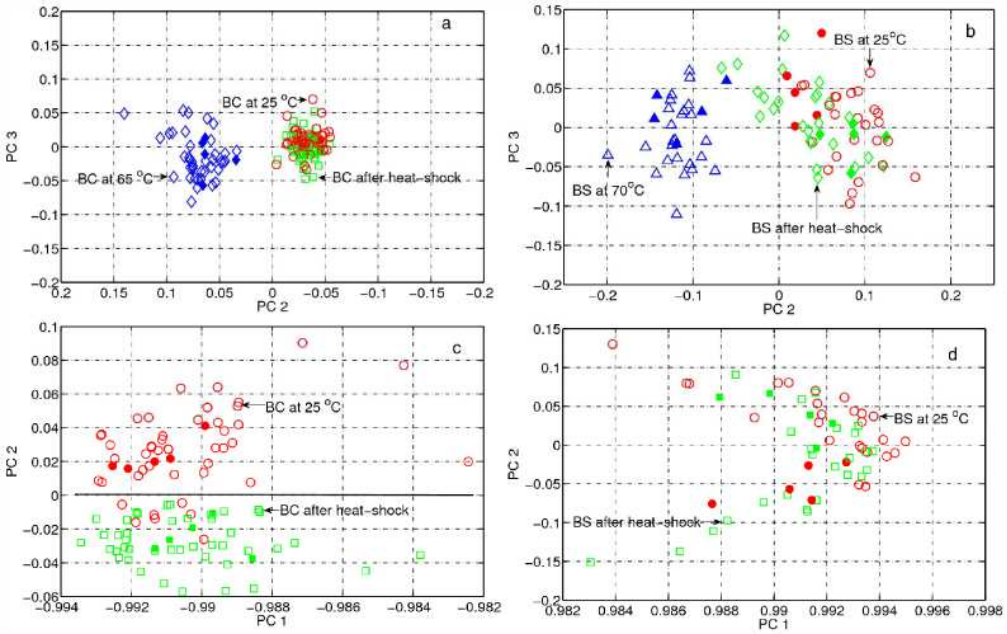

Fig. 3. Score plots of untreated spores (red), cooled heat-activated spores (green), and heat-activated spores held at the heat activation temperature (blue) based on non-polarized Raman spectra. The individual spores in training sets are represented with open symbols and the spores in testing sets with filled symbols, respectively. (a) *B. cereus*; (b) *B. subtilis*; (c) *B . cereus* with untreated and cooled heat-activated spores; (d) *B. subtilis* with untreated and cooled heat-activated spores . BC – *B. cereus*; BS – *B.subtilis*.

To determine if the minor spectral differences between individual untreated and cooled heat-activated spores can be distinguished, we ran the PCA analysis with the spectra of untreated and heat-activated spores, without including the data for heat-activated spores held at high temperature (Figs. 3(c) and 3(d)). The view angle was chosen along the PC1-PC2 axis in order to compare with the analysis based on polarized Raman spectra (Section 3.3). Figure 3(c) shows that for *B. cereus* spores, the untreated and cooled heat-activated spores were almost completely separated (Fig. 3(c)). The projection coefficients of the Raman spectra onto the second principle component (PC2) were mostly positive for untreated spores; however, these coefficients were negative for cooled heat-activated *B. cereus* spores. It should be noted that there was a small portion (<10%) of spores in the untreated group located in the region of the cooled heat-activated spores in the score plot of Fig. 3(c). This suggests that a small percentage of untreated spores were similar to heat-activated spores, and perhaps these spores had become activated without heat treatment, consistent with the low level of germination of these *B. cereus* spores without heat-activation noted above. Figure 3(d) shows that for *B. subtilis* spores, individual untreated spores and cooled heat-activated spores cannot be discriminated by non-polarized Raman spectroscopy. Note that a fairly large percentage (~30%) of untreated spores of *B. subtilis* were already activated, as noted above, as is commonly seen [34]. This could be one reason that the untreated and cooled heat-activated spores appear to overlap in Fig. 3(d). However, we will show that the scores in these two groups can still be discriminated by a "fine" polarized Raman spectroscopy (Section 3.3).

Figure 4 shows the first two loading spectra (PC1 and PC2) of the untreated and cooled heat-activated spores of *B. cereus* incubated at 25 °C, as well as their average Raman spectra. Figure 3(c) indicated that the differences between individual cooled heat-activated and

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untreated spores were mainly in PC2 (the second loading spectrum), as the untreated spores contained a larger amount of the PC2 component and the cooled heat-activated spores contained less. As shown in Fig. 4, the first principle component (PC1) was close to the average of the original spectra from the two groups and the second component (PC2) accounted for the variance between them. In the second loading spectrum (PC2), the peaks at 1014 cm^{-1} and 1446 cm^{-1} suggest that the state of Ca-DPA is not restored completely after the heated spores are cooled. In addition, there were two peaks at 1656 cm^{-1} and 1668 cm^{-1} in PC2, which are associated with the α-helical and nonregular structure of protein, respectively [29,30]. This suggests that the partial denaturation of protein during heat activation is also not completely reversed in the cooled heat-activated spores.

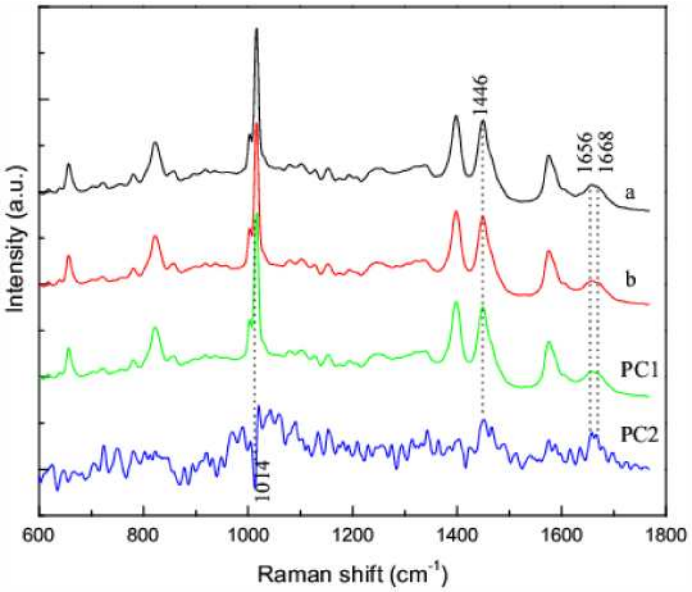

Fig. 4**.** Averaged Raman spectra for (a) untreated and (b) cooled heat-activated *B. cereus* spores and the loading spectra of PC1 and PC2.

3.3 Polarized Raman spectroscopy of single heat-activated spores of B.subtilis

For *B. subtilis* spores, we could not differentiate the cooled heat-activated and untreated spores (Section 3.2). However, polarized Raman spectroscopy might provide more information about structural differences between these spores, perhaps allowing them to be discriminated from each other. We measured the polarized Raman spectra of thirty individual spores for untreated spores at 25 °C, cooled heat-activated spores, and spores held at 70 °C. Figure 5 shows the parallel ($I_{//}$) and vertical components ($I_{\perp}$) of polarized Raman spectra of these spores, measured simultaneously (see Section 2.1), and shows that although the intensity of the parallel component is greater than that of the vertical component, the vertical component contains richer structural information.

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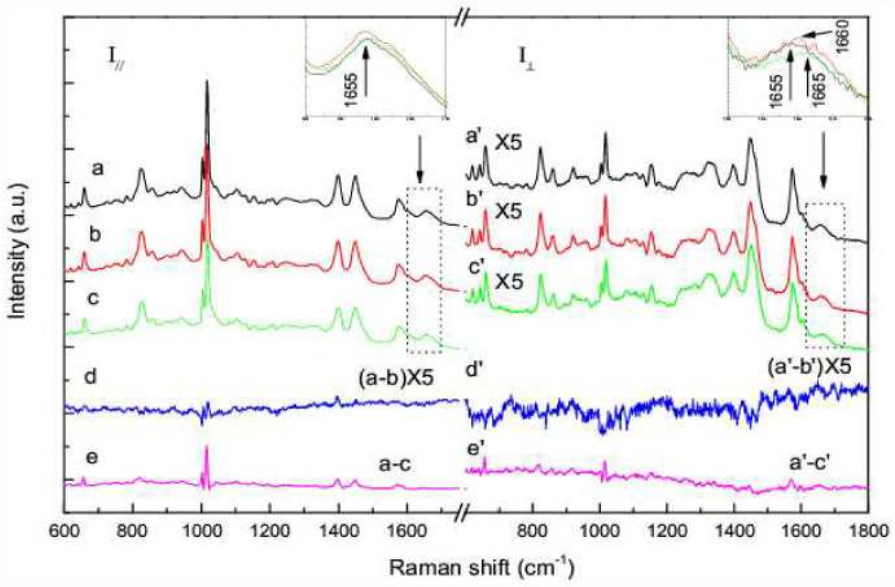

Fig. 5. Polarized Raman spectra of *B. subtilis* spores at 25 °C (a, a'); heat-activated spores held at either 25 °C (b, b') or at 70 °C (c, c'). The insets show the averaged spectra in the range of the protein amide I band. Curves d and d' are the subtractions of curve b (or b') from curve a (or a'), magnified by a factor 5 for display, and curves e and e' are the subtractions of curve c (or c') from curve a (or a'). Left: parallel components ($I_{//}$); Right: vertical components ($I_{\perp}$). All spectra were averaged over 30 spores, and the vertical baselines were shifted for display.

Table 3. Depolarization ratios of characteristic Raman bands of *B. subtilis* spores

	ρ_{824}	ρ_{1017}	ρ_{1395}	ρ_{1446}	ρ_{1572}
BS at 25 °C	0.23±0.01	0.086±0.005	0.13±0.02	0.34±0.02	0.52±0.02
BS after heat-shock	0.23±0.01	0.096±0.005	0.14±0.01	0.37±0.02	0.54±0.02
BS at 70 °C	0.25±0.02	0.11±0.01	0.18±0.02	0.43±0.02	0.56±0.02

Table 3 lists the depolarization ratios, defined as $\rho=I_{\perp}/I_{//}$, of several characteristic bands, where $I_{\perp}$ and $I_{//}$ are the intensities of the vertical and parallel polarization components, respectively. It can be seen that when heated to 70 °C, the depolarization ratios of bands at 1395 cm^{-1} and 1446 cm^{-1} were increased significantly. In addition, in the vertical components, the intensity of the band at 1655 cm^{-1} was decreased and the position of this band was shifted to 1665 cm^{-1}. However, these changes were not observed in the parallel components. This indicates that the partial denaturation of protein during heat activation is most likely reflected in the vertical component of polarized Raman spectra. After the heat-treated spores were cooled, the magnitude of difference spectra (curves d and d') from the untreated spores decreased and depolarization ratios returned close to the values of untreated spores. However, in the vertical components, the position of the amide I band returned to 1660 cm^{-1} for the cooled heat-activated spores, rather than to the 1655 cm^{-1} of the untreated spores. Note that reversion of heat-activated spores to the original untreated state generally takes many hours at 25 °C [4, 7], and thus the cooled heat-activated spores that we measured should still be heat-activated.

In order to determine if the vertical components of the polarized Raman spectra can be used to discriminate heat-activated from untreated *B. subtilis* spores, we also applied PCA to analyze the vertical spectra for spores held at 25 °C held at 25 °C after heat-activation, and during heat activation at 70 °C (Fig. 6(a)). As expected, the scores at 70 °C were separated completely from the other two groups by the second principle component (PC2). In addition, the untreated and cooled heat-activated spores could be partially separated by the third

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principle component (PC3). To discriminate these latter two groups further, we ran the PCA program without including the spores at 70 °C, and the scores were plotted in Fig. 6(b). In the new orthogonal basis, these two groups of spores were well separated from each other by the second principle component (PC2). PCA was also applied to the parallel polarized Raman spectra, but the results were similar to those based on non-polarization measurement (data not shown and Section 3.2).

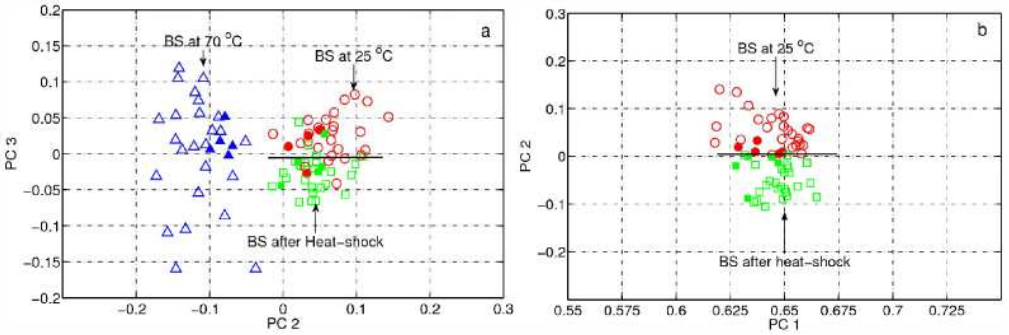

Fig. 6. (a) Score plots of *B. subtilis* spores either untreated (red) at 25 °C, heat-activated and held at 25 °C (green), and heat activated and held at 70 °C (blue) based on vertical components of polarized Raman spectra. The individual spores in training sets are represented with open symbols and the spores in testing sets with filled symbols, respectively. (b) Score plots of untreated spores of *B. subtilis* (red) and heat-activated spores held at 25 °C (green).

4. Conclusions

We have studied the heat activation of individual *B. cereus* and *B. subtilis* spores by non-polarized and polarized LTRS. We measured the Raman spectra of single untreated dormant spores, heat-activated spores after cooling, and spores held at the heat activation temperature. PCA was applied to discriminate these 3 groups of spores based on their Raman spectra. The results indicate that during heat activation the intensities and intensity ratios of Ca-DPA bands in spores were changed significantly for spores of *B. cereus* and *B. subtilis*. In addition, partial denaturation of protein was also observed in the Raman spectra. These changes became much smaller once the heat-activated spores were cooled, consistent with heat activation being a reversible process. For *B. cereus* spores, the minor spectral differences between the untreated and cooled heat-activated spores could be discriminated by PCA based on non-polarized Raman spectra. For *B. subtilis* spores, PCA based on non-polarized Raman spectra was incapable of discriminating the untreated and cooled heat-activated spores, but they could be distinguished by PCA based on polarized Raman spectra. These results reveal that some structural modifications in spore components caused by the sublethal heat activation process are not reversed when the heated spores are cooled, and perhaps it is these changes are crucial to allow heat-activated spores to germinate well in nutrient media. In addition, we demonstrated that the partial denaturation of spore proteins during heat activation is mainly reflected in the vertically polarized component instead of the parallel component in *B. subtilis* spores. Some of the spectral differences we saw may or may not be directly related to heat activation. However, it is gratifying that much of the change in bulk spore protein seen during heat activation is reversible. The technique developed in this work might provide a promising method to study the dynamic biochemical process of a single spore during heat-activation, a topic of interest to biologists. Moreover, compared to conventional germination procedures that take minutes to identify heat-activated spores in populations, our approach is able to identify a single heat-activated spore in seconds.

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