Real-time monitoring of *Bacillus subtilis* endospore components by attenuated total reflection Fourier-transform infrared spectroscopy during germination

Hon-Yeung Cheung, Jianxun Cui and Su-qin Sun†

Author for correspondence: Hon-Yeung Cheung. Tel: +852 2788 7746. Fax: +852 2788 7406.
e-mail: bhhonyun@cityu.edu.hk

Research Group for
Bioactive Products,
Department of Biology &
Chemistry, City University of
Hong Kong, Tat Chee Ave,
Hong Kong

Chemical changes of particular *Bacillus subtilis* spore components were monitored by attenuated total reflection Fourier-transform infrared spectroscopy (ATR/FTIR) during spore germination on a ZnSe internal reflection element. Within minutes of the initiation of spore germination, significant changes in the amount of calcium dipicolinate (DPA-Ca) and proteins were noted in the wild-type strain. The changes in a germination mutant (strain 1G9, *gerD*) were similar to those in the wild-type strain, but the rates of change were slower. The changes in another germination mutant (strain 1G7, *gerA*) were very different from those in the first two strains: germination was slow and incomplete, and proteins and DPA-Ca remained unaltered throughout the course of the germination study. This technique thus offers a sensitive and non-destructive method for real-time monitoring of various cellular components during spore germination.

Keywords: *Bacillus subtilis*, spore, germination mutants, ATR/FTIR

INTRODUCTION

Germination is a process by which a dormant spore develops into an actively growing vegetative cell. The steps involved in approximate temporal order are: activation, germination initiation, which includes loss of heat resistance, K^+ and Zn^{2+} fluxes, release of Ca^{2+} and dipicolinate (DPA) from the core followed by hydrolysis of cortex peptidoglycan, rehydration of the core protoplast, and resumption of metabolic activity (Dring & Gould, 1971). Many pleiotropic mutants unable to germinate are defective either in coat structure or in cortex structure. These mutants are blocked at various stages in the germination process (Moir, 1992).

Spore germination has been extensively studied in a number of bacteria, with recent studies of the mechanism focusing on the contribution of biochemical and genetic factors (Keynan, 1978; Jenkinson & Loard,

1983; Cheung & Brown, 1985; Jenkinson, 1988; Preston & Douthit, 1989; Moir & Smith, 1990; Johnstone, 1994; Moir *et al.*, 1994; Kuhn *et al.*, 1995; Cheung *et al.*, 1998). Some quantifiable events during germination, such as the loss of heat resistance, the release of DPA and hexosamine-containing cortex fragments, and the change from phase-bright to phase-dark under a phase-contrast microscope, have been described. The loss of heat resistance can be measured by plate counts, and a spectrophotometric assay can be used to measure the release of DPA and Ca^{2+} (Moir, 1992). However, germination is so rapid that it is not clear whether these events precede other changes associated with initiation of germination. New methods capable of real-time measurement of spore germination are needed.

Attenuated total reflection Fourier-transform infrared spectroscopy (ATR/FTIR) is a sensitive technique providing structural information at the molecular level. Some cellular components of bacteria can be identified by infrared spectroscopy (Helm *et al.*, 1991; Naumann *et al.*, 1991), and constituents such as capsules, DPA and intracellular materials have been detected with this method (Norris & Greenstreet, 1958; Helm & Naumann, 1995). Therefore it may offer a method for obtaining useful chemical information during spore

† **Present address:** Analysis Centre, Tsinghua University, Beijing, 100084, China.

Abbreviations: ATR/FTIR, attenuated total reflection Fourier-transform infrared spectroscopy; DPA, dipicolinate; DPA-Ca, calcium dipicolinate.

Fig. 1. Germination of spores of *B. subtilis*. Spores of the wild-type strain (curve A), the *gerD* mutant (curve B) and the *gerA* mutant (curve C) were induced to germinate in nutrient broth at room temperature as described in Methods. The extent of germination was assessed at 5 min intervals by measuring the decrease in OD₆₀₀ of the spore suspension.

germination in real time. The aim of this work was to detect by ATR/FTIR in intact spores the changes in protein, DPA-Ca and polysaccharide that occur during germination of *Bacillus subtilis* spores.

METHODS

Bacterial strains. A wild-type strain of *Bacillus subtilis* was originally obtained from Ernst Freese at the National Institutes of Health, Bethesda, MD, USA; it was designated as 60015 by NIH. Two germination mutants, 1G7 (*gerA11 thr5 trpC2*) and 1G9 (*gerD19 trpC2*) of *B. subtilis* were obtained from the Bacillus Genetic Stock Center, Ohio State University, Columbus, USA.

Preparation of spores. Cells of *B. subtilis* were incubated at 37 °C for 14–16 d in a modification of the nutrient sporulation medium of Vasantha & Freese (1979). The medium contained nutrient broth (Oxoid) 8 g dry base l⁻¹, Na₂HPO₄/KH₂PO₄ (67 mM P_i, pH 7.0), 1 mM MgCl₂, 0.7 mM CaCl₂, 50 µM MnCl₂ and 1 µM FeCl₃. Spores were harvested and repeatedly washed with phosphate-buffered saline (PBS) until over 95% of the cells were free spores. A final spore concentration of approximately 1–5 × 10⁸ spores ml⁻¹ was reconstituted in PBS and kept at 4 °C until used.

Identification of particular spore constituents by FTIR spectroscopy. A weighed amount of spore pellet spun down from the spores in PBS was added to distilled water to give a final concentration of about 30 mg ml⁻¹. Typically, 20 µl of this spore suspension was spread uniformly on a CaF₂ crystal plate and dried to a film under moderate vacuum (2.5–7.5 kPa). The crystal plate was placed on a sample holder in the sample compartment of a FTIR spectrometer (Spectrum 2000, Perkin Elmer), equipped with a DTGS (deuterated triglycine sul-

Fig. 2. Deconvoluted FTIR spectra of spores of the three strains of *B. subtilis*. Washed dormant spores were loaded onto a CaF₂ crystal plate and freeze-dried under moderate vacuum. Deconvoluted FTIR spectra of the dormant spores of the wild-type strain (curve A); the *gerD* mutant (curve B) and the *gerA* mutant (curve C) were derived from the original FTIR spectra obtained directly from the FTIR spectrometer as described in Methods.

phate) detector in the 375–2000 cm⁻¹ range with a nominal resolution of 4 cm⁻¹. Spectra were recorded at room temperature relative to a sample-free crystal, and were the means of 50 scans. The resolution of the spectra was subsequently enhanced by applying a self-deconvolution program, Spectrum version 2.01 (Perkin-Elmer). This software program is analogous to a deconvolution method developed by Kauppinen *et al.* (1981). Peak positions and spectral resolution were significantly improved by modifying two adjustable parameters, gamma and length, as specified in the software, where gamma is the line-narrowing factor and length is the smoothing applied to limit the noise generated by line-narrowing. Good peak positions of the deconvoluted spectra were observed at gamma value = 4 and smoothing length = 50.

Germination study. Germination of the spore suspensions was monitored by measuring the decrease in OD₆₀₀ (Cheung *et al.*, 1998). Once the spores were mixed with nutrient broth, germination was monitored at 37 °C and at room temperature (20 °C) by measuring OD₆₀₀ at 5 min intervals over a 60 min period. The rate of germination was confirmed by counting the proportion of phase dark/bright spores. Changes in OD₆₀₀ were plotted relative to the original OD₆₀₀ of the control sample. Each data point was the mean of at least two separate experiments.

Fig. 3. Dynamic changes in the FTIR spectra of germinating spores. An aliquot of spore suspension of *B. subtilis* in nutrient broth was spread on a ZnSe internal reflection element and the spores were induced to germinate at room temperature as described in Methods. FTIR spectra were taken at 2 min intervals when the spores were germinating. (a) Wild-type strain; (b) *gerD* mutant; (c) *gerA* mutant.

Real-time monitoring of chemical components by ATR/FTIR during spore germination. Spores of *B. subtilis* from the stock were suspended in 1 ml nutrient broth. About 0.5 ml of the

resuspended spores was immediately put onto the ZnSe internal reflection element. The element was then placed on the ATR accessory and positioned on an optical bench inside

the FTIR spectrometer equipped with a Ge/KBr beam splitter and DTGS detector. Incubation was carried out at room temperature. The spectrum of one ZnSe internal reflection element served as a reference, and that of another covered with nutrient broth was used for subtraction from the sample spectra. Spectral resolution was 4 cm^{-1} and 20 scans were recorded for each spectrum. During germination, spectra were collected at intervals of about 2 min. The spectra were processed by the ATR correction accessory of the Spectrum version 2.01 software. This allows spectra of different types to be compared and yet does not affect qualitative interpretation. It consists of multiplying the absorbance values by a factor f_i that varies with the wavelength λ_i and the contact correction factor C according to $f_i = (\lambda_{\min} - C)/(\lambda_i - C)$, where $\lambda_{\min}$ is the shortest wavelength in the spectrum. The reason for this is to leave the absorbance value at the shortest wavelength unchanged. As the contact term is increased, bands at longer wavelengths are increasingly attenuated relative to those at shorter wavelengths. Throughout this work, a contact correction factor equal to zero was used.

RESULTS AND DISCUSSION

Spore germination monitored by optical density

The germination curves for the three strains of *B. subtilis* in nutrient broth at room temperature (20°C) are shown in Fig. 1. Decreases in optical density are indicative of the proportion of spores that have germinated (Cheung *et al.*, 1998). As shown by the extent of phase-darkening, spores of the germination mutants were slower to germinate than the wild-type (Moir, 1992). The germination rate of spores of the *gerA* mutant was significantly slower than that of wild-type spores, while spores of the *gerD* mutant germinated very slowly.

Identification of particular spore constituents by FTIR spectra

Consistently reproducible FTIR spectra were obtained with different batches of dormant spores of each of the three strains tested and also with spores of *Bacillus stearothermophilus* (data not shown). Fig. 2 shows the deconvoluted spectra of spores of the wild-type strain and of the two germination mutants of *B. subtilis*. The absorption peaks at $1652\text{--}1654$, $1541\text{--}1543$ and $1277\text{--}1279\text{ cm}^{-1}$ were assigned to the amide I, II and III bands of proteins respectively (Byler & Susi, 1986), representing the C=O stretching mode and the N-H deformation modes of the secondary amide structure. The peak at $1379\text{--}1380\text{ cm}^{-1}$ was due to symmetric stretching vibrations of the carboxyl group of DPA chelated to Ca^{2+} (Helm & Naumann, 1995). An additional band at $1566\text{--}1570\text{ cm}^{-1}$ was due to the C-N vibrations of the DPA ring. The band near 1443 cm^{-1} demonstrates the existence of an acid peptide, most probably glutamate and 3-phospho-D-glycerate (Helm & Naumann, 1995). The absorption peak at 1082 cm^{-1} was attributed to the ring vibrations of polysaccharide (Cheung *et al.*, 1998).

Although the FTIR spectra for all three strains were very

similar, the wild-type and mutants did show some minor differences in the relative intensities and positions of the peaks. The spectra in Fig. 2 imply differences in the relative protein, DPA-Ca and polysaccharide content of the spores of the strains. The ratio of peak heights at wavenumbers 1379 and 1443 cm^{-1} was higher in spectra of the *gerA* mutant spores than in those of the other two strains. This indicates that DPA-Ca was more abundant in spores of this mutant than in the other two strains. The band at 1655 cm^{-1} , which is typical of α -helical structures, was more abundant in the *gerD* than in the *gerA* mutant or the wild-type. The polysaccharide absorbance peaks in the spores of both germination mutants were shifted 6 wavenumbers to lower values than in the wild-type. Since the downward shift of an FTIR peak is an indication of increased co-ordination (Liedberg *et al.*, 1987), this suggests that the molecular structure of a polysaccharide, most probably the peptidoglycan, in the germination mutants was more stable and regular than that in the wild-type.

Dynamic changes in the FTIR spectra of germinating spores

Structural changes of spore components during germination were monitored for 1 h in real time by ATR/FTIR. Fig. 3(a-c) shows the three-dimensional maps of FTIR spectra of germinating spores of the three *B. subtilis* strains. The increasing absorbance of some functional groups and conformational structures seen in Fig. 3 indicates that their amounts inside the spores increased after exposure to germinants. Although all increased in amount, some did so faster than others. An increase in the intensity of an absorbance peak could have three causes: gradual deposition of spores on the ZnSe internal reflection element, a structural change in the spores during germination, and probably evaporation of water during the experiment.

The changes of spore components can be observed in Fig. 4(a-c), which shows FTIR spectra of germinating spores of *B. subtilis* at the start, and at 15 min and 1 h after the start of germination. There was a shift of up to 8 cm^{-1} for some bands in the spectra of the wild-type strain: from 1443 and 1384 cm^{-1} at the start to 1451 and 1388 cm^{-1} , respectively, at 1 h. Comparable shifts of up to 7 cm^{-1} were observed in the *gerD* mutant: from 1443 and 1384 cm^{-1} at the start to 1450 and 1388 cm^{-1} , respectively, at 1 h. However, in the spectra of the *gerA* mutant, no shifts were observed for these peaks. Comparing the relative peak heights at different times of germination with those at the start of the experiment provides information about the rates of change in each component. Within 15 min of the initiation of spore germination, the amount of protein (1279 cm^{-1}) and chelation state of DPA to Ca^{2+} (1380 cm^{-1}) were estimated to have changed by 26% and 11%, respectively, in the wild-type strain. After 1 h incubation, the amount of protein and the chelation state of DPA had changed by 34% and 17%, respectively. Changes in the *gerD* mutant spores were similar to those in the wild-

type spores except that they were slower. The chemical changes in the *gerA* mutant were very different from those in the other two strains. Modification of protein and release of DPA-Ca were not observed in the first 1 h in this germination mutant. This confirms the *gerA* mutant's inability to break its dormant state reported previously and matches our optical density measurements.

The above interpretation is supported by the relative intensity changes of the C=O anti-symmetric stretching mode relative to that of the neighbouring amide I band near 1650 cm^{-1} in the FTIR spectra. The intensity of this band, near 1620 cm^{-1} in Fig. 4(a), decreased markedly for the wild-type from the start to 15 min, but it showed no apparent change for the *gerA* mutant (Fig. 4c) and changed only slightly for the *gerD* mutant (Fig. 4b).

Degradation of spore protein during germination provides an important source of monomeric substrates for protein synthesis and ribonucleotide metabolism at subsequent stages (Setlow, 1970). The FTIR spectra showed that protein modifications began immediately following the initiation of germination, and approximately 26% of the spore protein was hydrolysed in the first 15 min in the wild-type spores. These results are consistent with other authors' observations that about 15–20% of the total protein in spores was degraded in the first 20 min of germination (Setlow, 1983).

The structural changes in DPA-Ca are normally accompanied by the release of equimolar amounts of Ca^{2+} and DPA by a germinating spore (Dring & Gould, 1971). In the past, changes in these molecules have been monitored by differential cathode-ray polarography, which is cumbersome and time-consuming. ATR/FTIR spectroscopy, on the other hand, can provide real-time monitoring of the molecules in a germinating spore. From the intensity of a particular absorbance band in the IR spectra, changes in the functional groups of molecules inside the cells can be followed dynamically in real time and *in situ*. Thus this method has substantial value for monitoring spore components during germination and for characterizing strains.

ACKNOWLEDGEMENTS

The authors would like to thank City University of Hong Kong for providing a studentship to Mr Jianxun Cui. They also thank Otto Shek and Sam Li of Perkin Elmer (Hong Kong) Ltd for use of their IR facilities. Their generous help is wholeheartedly acknowledged. The authors are grateful to the editors and the anonymous referees for constructive comments for the improvement of the manuscript.

Fig. 4. FTIR spectra of spores of *B. subtilis* germinating in nutrient broth: at the start (curve A), at 15 min (curve B) and at 1 h (curve C). (a) Wild-type strain; (b) *gerD* mutant; (c) *gerA* mutant.

REFERENCES

- Byler, D. M. & Susi, H. (1986). Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* **25**, 469–487.
- Cheung, H. Y. & Brown, M. R. W. (1985). Coat structure and morphogenesis of bacterial spores in relation to the initiation of spore germination. In *Fundamental and Applied Aspects of Bacterial Spores*, pp. 227–239. Edited by G. J. Dring, D. J. Ellar & G. W. Gould. New York: Academic Press.
- Cheung, H. Y., So, C. W. & Sun, S. Q. (1998). Interfering mechanism of sodium bicarbonate on spore germination of *Bacillus stearothermophilus*. *J Appl Microbiol* **84**, 619–626.
- Dring, G. J. & Gould, G. W. (1971). Sequence of events during rapid germination of spores of *Bacillus cereus*. *J Gen Microbiol* **65**, 101–104.
- Helm, D. & Naumann, D. (1995). Identification of some bacterial cell components by FTIR spectroscopy. *FEMS Microbiol Lett* **126**, 75–80.
- Helm, D., Labischinski, H., Schallehn, G. & Naumann, D. (1991). Classification and identification of bacteria by Fourier-transform infrared spectroscopy. *J Gen Microbiol* **137**, 69–79.
- Jenkinson, H. F. (1988). Altered arrangement of proteins in the spore coat of a germination mutant of *Bacillus subtilis*. *J Gen Microbiol* **129**, 1945–1958.
- Jenkinson, H. F. & Loard, H. (1983). Protease deficiency and its association with defects in spore coat structure, germination and resistance properties in a mutant of *Bacillus subtilis*. *J Gen Microbiol* **129**, 2727–2737.
- Johnstone, K. (1994). The trigger mechanism of spore germination: current concepts. *J Appl Bacteriol Symp Suppl* **76**, 17S–24S.
- Kauppinen, J. K., Moffat, D. S., Mantsch, H. H. & Cameron, D. (1981). Fourier self-deconvolution: a method for resolving intrinsically overlapped bands. *Appl Spectrosc* **35**, 217–227.
- Keynan, A. (1978). Spore structure and its relations to resistance, dormancy and germination. In *Spores VII*, pp. 43–53. Washington, DC: American Society for Microbiology.
- Kuhn, N. J., Setlow, B., Setlow, P., Cammack, R. & Williams, R. (1995). Cooperative manganese(II) activation of 3-phosphoglycerate mutase of *Bacillus megaterium*: a biological pH-sensing mechanism in bacterial spore formation and germination. *Arch Biochem Biophys* **320**, 35–42.
- Liedberg, B., Carlsson, C. & Lundstrom, I. (1987). An infrared reflection-absorption study of amino-acids adsorbed on metal surface: L-histidine and L-phenylalanine on gold and copper. *J Colloid Interface Sci* **120**, 113–124.
- Moir, A. (1992). Spore germination. In *Biology of Bacilli*, pp. 23–38. Edited by R. H. Doi & M. McGloughlin. London: Butterworth-Heinemann.
- Moir, A. & Smith, D. A. (1990). The genetics of bacterial spore germination. *Annu Rev Microbiol* **44**, 531–553.
- Moir, A., Kemp, E. H., Robinson, C. & Corfe, B. M. (1994). The genetic analysis of bacterial spore germination. *J Appl Bacteriol Symp Suppl* **76**, 9S–16S.
- Naumann, D., Helm, D. & Labischinski, H. (1991). Microbiological characterizations by Fourier-Transform infrared spectroscopy (FT-IR). *Nature* **351**, 81–82.
- Norris, K. P. & Greenstreet, J. E. S. (1958). On the infrared absorption spectrum of *Bacillus megaterium*. *J Gen Microbiol* **19**, 566–580.
- Preston, R. A. & Douthit, H. A. (1989). Germination of *Bacillus cereus*: critical control by L-alanine racemase. *J Gen Microbiol* **130**, 3123–3133.
- Setlow, P. (1970). Biochemical studies of bacterial sporulation and germination. XXIII. Nucleotide metabolism during spore germination. *J Biol Chem* **245**, 3645–3652.
- Setlow, P. (1983). Germination and outgrowth. In *The Bacterial Spore II*, pp. 211–225. Edited by A. Hurst & G. W. Gould. New York: Academic Press.
- Vasantha, N. & Freese, E. (1979). The role of manganese in growth and sporulation of *Bacillus subtilis*. *J Gen Microbiol* **112**, 329–336.

Received 2 April 1998; revised 23 December 1998; accepted 25 January 1999.