Uptake and levels of the antibiotic berberine in individual dormant and germinating *Clostridium difficile* and *Bacillus cereus* spores as measured by laser tweezers Raman spectroscopy

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Objectives: Spores of *Clostridium difficile* and *Bacillus cereus* are major causes of nosocomial diarrhoea and foodborne disease. Our aim was to measure the dynamics of the uptake of the antibiotic berberine by individual germinating spores and the levels of berberine accumulated in germinated spores.

Methods: Laser tweezers Raman spectroscopy (LTRS) and differential interference contrast microscopy were used to measure levels of berberine accumulated in single germinating spores and to monitor berberine uptake and germination of individual *C. difficile* and *B. cereus* spores.

Results: MIC values of berberine for *C. difficile* and *B. cereus* spores were 640 and 256 mg/L, respectively. Levels of berberine accumulated at the berberine MICs in individual germinated spores were heterogeneous, with values of 17.1 ± 5.4 and 12.7 ± 5.5 g/L for *C. difficile* and *B. cereus* spores, respectively. These values were 25–50-fold higher than the MIC values. However, berberine did not affect the germination of *C. difficile* and *B. cereus* spores, but did block germinated spores' outgrowth. Berberine uptake kinetics were similar for these two kinds of spores. After the addition of germinants, berberine began to enter germinating spores at the time (T_{lag}) when rapid release of the spore core's large depot of the 1:1 chelate of Ca^{2+} with dipicolinic acid began, and the level of berberine taken up was maximal shortly after spore cortex lysis was completed (T_{lysis}).

Conclusions: LTRS can be used to measure uptake and levels of berberine in single cells. High levels of berberine can enter spores of *C. difficile* and *B. cereus* soon after germination is initiated, thus inhibiting spore outgrowth and minimizing hazards posed by germinated spores.

Introduction

In recent years, there has been much study of Gram-positive spore-forming bacteria of *Clostridium* and *Bacillus* species, in part because spores of some of these species are human pathogens and some cause food spoilage and foodborne diseases. For example, spores of *Clostridium difficile* are a leading cause of antibiotic-associated diarrhoea worldwide, and some strains of *Bacillus cereus* spores are harmful to humans and cause foodborne illness.[1–3] A major reason for the importance of spores in the deleterious effects of these organisms is that spores can remain dormant for long periods and are extremely resistant to all manner of stresses, including antibiotics and many commonly used disinfectants.[4,5] For example, *B. cereus* foodborne illnesses occur primarily due to survival of the bacterial spores when food is improperly cooked, as cooking temperatures $\leq 100°C$ allow some *B. cereus* spores to survive.[6] Importantly, under appropriate conditions spores can rapidly return to life in the process of germination followed by outgrowth and vegetative growth, and can cause deleterious effects likely during both of the latter processes. However, during germination and outgrowth, spores lose their resistance properties, initiate metabolism and macromolecular synthesis, and become relatively easy to kill.[1,7] Thus, to minimize potential hazards of germinated spores, it would be helpful to understand spore germination and, in the case of use of antibiotics for spore control, to know when and how much antibiotic is taken up by spores during their germination and outgrowth, since antibiotics are unlikely to penetrate the spore core when the spores are in their dormant state.

In an era with frequent occurrence of antibiotic-resistant bacteria, the bioactive compounds in plants have been a focus of renewed attention due to their novel antimicrobial properties and a long history of use.[8] Among such antibiotics is berberine, a well-known natural isoquinoline alkaloid isolated primarily

from various plant species, including those of the genera *Berberis*, *Mahonia* and *Coptis*.[9] This compound has been used as a broad-spectrum antimicrobial agent for many years in traditional Chinese, Native American and Western medicine.[10] Berberine has been shown to exhibit activity against viruses, fungi, protozoans, helminths and a variety of bacteria, including many pathogenic species and MDR strains of *Mycobacterium tuberculosis* and MRSA.[11,12] Berberine's antibacterial activity has been ascribed to effects on cell membranes, interactions with DNA and inhibition of cell division.[13–15] In addition, berberine has been shown to exhibit tumoricidal and anti-inflammatory activities.[16,17] However, the effects of berberine on spores of pathogenic spore-forming bacteria have not been well studied. It is likely that berberine will not enter the core of dormant spores due to the core's extreme impermeability, consistent with dormant spores' resistance to antibiotics.[7] It is, however, less clear whether berberine will enter spores during their germination and thus inhibit spore outgrowth and gene expression during this period. Additionally, growth-inhibitory berberine levels accumulated in single cells and the kinetics of berberine uptake in single cells have not been determined for *C. difficile* and *B. cereus* due to the lack of non-invasive single-cell analysis techniques.

In this work, laser tweezers Raman spectroscopy (LTRS) and differential interference contrast (DIC) microscopy were used to analyse levels and uptake kinetics of berberine in individual dormant and germinating *C. difficile* and *B. cereus* spores. The advantages of using LTRS for analysis of berberine in single spores include: (i) single-cell Raman spectroscopy allows non-invasive and direct quantification of berberine levels accumulated in individual spores because berberine has specific Raman peaks at 1518 and 1397 cm^{-1}; (ii) a major spore small molecule, the 1:1 chelate of Ca^{2+} and dipicolinic acid (CaDPA), also gives a number of specific Raman peaks, such that CaDPA levels in individual spores can be determined by the intensity of the CaDPA-specific Raman band at 1017 cm^{-1};[18] (iii) since CaDPA release is a major event in spore germination, measurement of CaDPA release and berberine uptake kinetics simultaneously will allow placement of berberine uptake in the overall germination pathway; (iv) optical tweezers can hold an individual spore in aqueous germination media for 1–2 h with a low-power near-infrared laser, so that Raman spectroscopy and DIC microscopy can determine the berberine and CaDPA levels in the trapped spore in real time;[19] and (v) single-cell measurements allow characterization of cellular heterogeneity in levels and uptake kinetics of berberine among a population of germinating spores.[20,21] The results of these analyses might lead to regimens that could minimize the deleterious effects of these potentially harmful spores.

Materials and methods

Bacterial strains and spore preparation

Berberine chloride was purchased from ChemBridge (San Diego, CA, USA). The bacterial strains used in this work were *C. difficile* ATCC 43593 with the PCR ribotype 060[22] and *B. cereus* T. Spores of *C. difficile* were prepared on 70:30 sporulation medium agar and were harvested, purified and stored as described previously.[23] *B. cereus* spores were prepared and purified as described previously.[24] All spores used in this study were >98% free of sporulating cells, germinated spores and debris, as observed by phase-contrast microscopy.

MIC measurements

The antimicrobial activity of berberine starting from dormant spores was determined in triplicate by analysis of cell growth with various berberine concentrations. Spores (10^5 cfu/mL) were inoculated into 1 mL of appropriate medium in test tubes with serial 2-fold dilutions of berberine. *C. difficile* spores were inoculated into BHIS medium (brain heart infusion, Sigma–Aldrich, St Louis, MO, USA) plus 0.1% taurocholate as a germinant, and *B. cereus* spores were inoculated into LB medium plus 10 mM L-valine as a germinant. *C. difficile* was cultured at 37°C in a BD GasPak Jar (Fisher Scientific, Suwanee, GA, USA), which was used to create an anaerobic environment, and *B. cereus* was cultured aerobically at 37°C with shaking. After 18 h of incubation, the optical density at 600 nm (OD_{600}) of the cultures was measured. The MIC values were defined as the minimum concentration of berberine that allowed no visible cell growth from spores after 18 h in the test tubes. Phase-contrast microscopy was used to examine the size and shape of the germinated spores at the end of cell growth to determine whether outgrowth of germinated spores was also inhibited by berberine.

Spore germination and berberine level measurements in single germinated and dormant spores

Prior to germination, *C. difficile* spores were not heat shocked, since heat activation is not needed to stimulate *C. difficile* spore germination.[25] *B. cereus* spores were heat activated in water by incubation at 65°C for 30 min and then cooling on ice for at least 15 min before initiating spore germination. For determination of berberine levels in individual germinating spores, *C. difficile* spores (10^7 cfu/mL) were routinely germinated aerobically at 37°C with 0.25% taurocholate and 15.6 mM glycine in BHIS medium with various concentrations of berberine for 60 min, as an anaerobic environment is not needed for *C. difficile* spores to complete germination.[25] *B. cereus* spores (10^7 cfu/mL) were germinated at 37°C with 10 mM L-valine in LB medium with various concentrations of berberine for 60 min. Dormant *C. difficile* and *B. cereus* spores (10^7 cfu/mL) were incubated at 37°C in 10 mM Tris–HCl (pH 7.4)–150 mM NaCl and 25 mM K-HEPES (pH 7.4) buffer, respectively, in combination with various berberine concentrations. Berberine levels in individual spores were determined by LTRS as described previously.[19,20] Briefly, individual dormant or germinated spores were randomly optically trapped by a 780 nm laser beam with a power of 20 mW, and Raman scattering excited by the same laser beam was measured with a charge-coupled device (CCD) coupled with a spectrograph. The spores' berberine content was determined from the intensities of their berberine-specific Raman band at 1518 cm^{-1} compared with the intensities of this band from berberine solutions of known concentration (see Results section, curve e in Figure 2).

Berberine uptake during spore germination

Individual spores were optically trapped by laser tweezers with a very low power (2–3 mW) and the kinetics of spore germination and berberine uptake were measured simultaneously by DIC microscopy and Raman spectroscopy as described previously.[19,21] The use of this low power of the near-infrared laser for optical trapping and the excitation of Raman spectroscopy was to reduce the potential effect of the laser on germination of the confined spore held in aqueous solution for a long time period.[19] The time-lapse Raman spectra were acquired at a rate of 60 s per spectrum for 60 min. CaDPA and berberine levels in individual trapped spores were determined from the intensities of CaDPA- and berberine-specific Raman bands at 1017 and 1518 cm^{-1}, respectively. The DIC images were recorded at a rate of 1 frame per 5 s for 60 min by a digital CCD camera (16 bits; 1600×1200 pixels) and analysed with the Matlab program to calculate the spores' DIC image intensity. As described previously,[19–21] the end of the rapid fall in the spores' DIC image intensity

during spore germination precisely corresponded to the point at which release of CaDPA was complete, and this time was defined as $T_{release}$. At this time, the DIC image intensity (I) was 30%–35% of that at the first time of measurement (T_0), when the image intensity at T_0 (corresponding to that of the dormant spore) was set at 1 and the DIC image intensity at the end of measurements (corresponding to that of the fully germinated spore) was set at 0. The CaDPA content of spores at T_0 was set at 1 and the content at the end of measurements was set at 0, since the DIC intensity was nearly coincident with the CaDPA level prior to $T_{release}$. In addition to $T_{release}$, the parameters T_{lag}, the time when rapid CaDPA release began, and T_{lysis}, the time when spores' peptidoglycan cortex hydrolysis was complete, were also used to describe the kinetics of the germination of individual spores, as described previously.[20] In order to compare CaDPA release and berberine uptake kinetics, the berberine content in spores at the end of measurements or the maximum value was arbitrarily set at 1.

Results

Determination of berberine MIC for cell growth from C. difficile and B. cereus spores

With increasing berberine concentrations in media supporting spore germination, spore outgrowth and cell growth, there was increasing inhibition of cell growth from dormant spores (Figure 1). Cell growth from C. difficile and B. cereus spores was completely inhibited at berberine concentrations of 640 and 256 mg/L, respectively indicating that berberine MIC values for C. difficile and B. cereus growth from spores are 640 and 256 mg/L, respectively. In addition, examination by phase-contrast microscopy of individual spores at the end of growth experiments showed that at the berberine MIC value almost all C. difficile and B. cereus spores had turned phase dark, but had neither swelled nor elongated (data not shown), indicating that these spores had germinated but their outgrowth was inhibited by berberine.
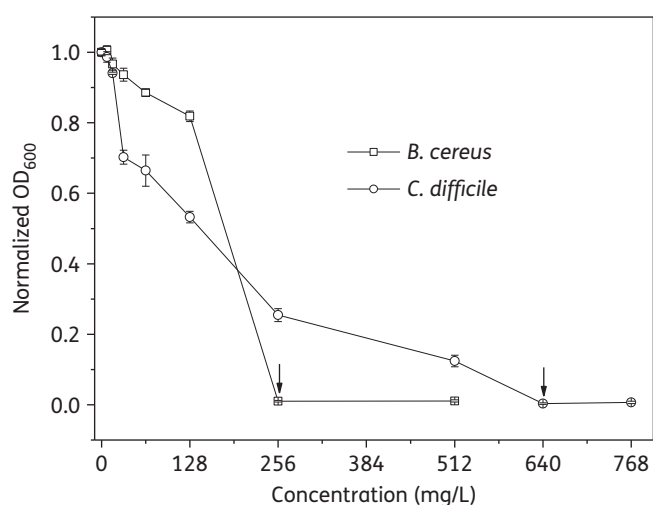

Figure 1. Determination of berberine MIC values for cell growth from C. difficile and B. cereus spores. Spores (10^5 cfu) were inoculated into 1 mL of BHIS medium plus 0.1% taurocholate (C. difficile) or LB medium plus 10 mM L-valine (B. cereus) plus serial 2-fold dilutions of berberine, and cultures were grown as described in the Materials and methods. After 18 h, the OD_{600} value was measured and normalized to the control sample without berberine addition. The MIC (vertical arrows) was defined as the minimum concentration of berberine at which no cell growth was observed in the test tube after 18 h.

Levels of berberine taken up by individual spores

Since berberine is able to inhibit outgrowth of spores, obvious questions are whether and how much berberine is taken up into spores during this period. In order to answer this question, a method is needed to measure berberine concentrations in individual spores. Thus, we tested whether LTRS is suitable for this purpose. First, we compared Raman spectra of germinated and dormant spores incubated with or without berberine, as well as that of berberine alone (Figure 2). Berberine alone showed two obvious large Raman spectral peaks at 1397 and 1518 cm^{-1} (Figure 2, curve e). As described previously, the high levels of CaDPA in the dormant spore core result in multiple major Raman peaks at 1017, 1395, 1447 and 1572 cm^{-1} (Figure 2, curve a).[18,19] Spores germinated without berberine present lacked the CaDPA-specific Raman peaks (Figure 2, curve c), since the spores' large CaDPA depot is excreted during spore germination.[20,26] The average Raman spectra of 30 individual spores germinated in the presence of berberine resulted in large berberine-specific peaks at 1397 and 1518 cm^{-1}, while the average Raman spectra of 30 individual dormant spores incubated with berberine had only very small peaks at 1397 and 1518 cm^{-1} (Figure 2, curves b, d and e). These results suggest that while much berberine can be accumulated in germinated spores, only minimal levels are accumulated in dormant spores. Together, the results noted above show that berberine in individual germinated or dormant spores has an obvious specific Raman peak at 1518 cm^{-1}. Consequently, in further experiments berberine
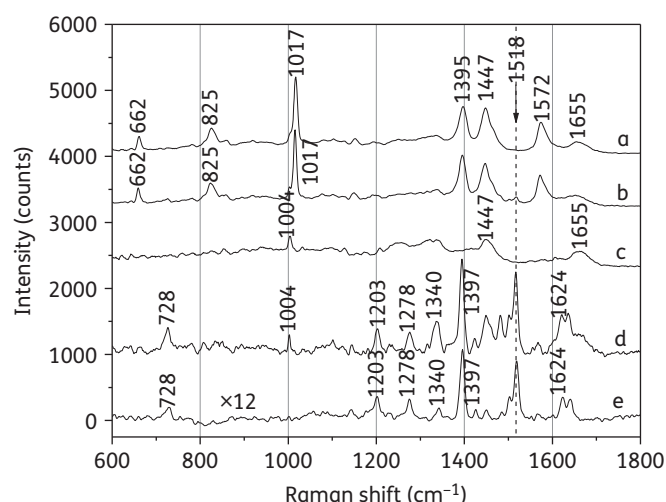

Figure 2. Average Raman spectra of 30 individual C. difficile dormant and germinated spores incubated with or without berberine. Raman spectra for individual spores were acquired as described in the Materials and methods with an integration time of 30 s, and spectra for 30 individual spores of each type were averaged. Spores were germinated in BHIS medium plus germinants as described in the Materials and methods and dormant spores were also incubated as described in the Materials and methods. (a) Dormant spores incubated without berberine in buffer. (b) Dormant spores incubated with 500 mg/L berberine for 60 min. (c) Germinated spores incubated without berberine. (d) Germinated spores incubated with 500 mg/L berberine. (e) Raman spectrum of 500 mg/L berberine in 10 mM Tris–HCl (pH 7.4)–150 mM NaCl. Note that the Raman intensity in curve (e) is magnified by a factor of 12 for display. The arrow and dashed line indicate the berberine-specific Raman band at 1518 cm^{-1} used to determine the berberine levels in individual spores.

levels in individual spores were determined from the intensities of the berberine-specific Raman band at 1518 cm^{-1} in spores and calibrated by the intensities of this same Raman band from solutions of known berberine concentration.

Berberine accumulation in individual germinated or dormant *C. difficile* and *B. cereus* spores

Berberine's MIC value for growth from *C. difficile* spores is higher than that for *B. cereus*. However, whether the MIC berberine
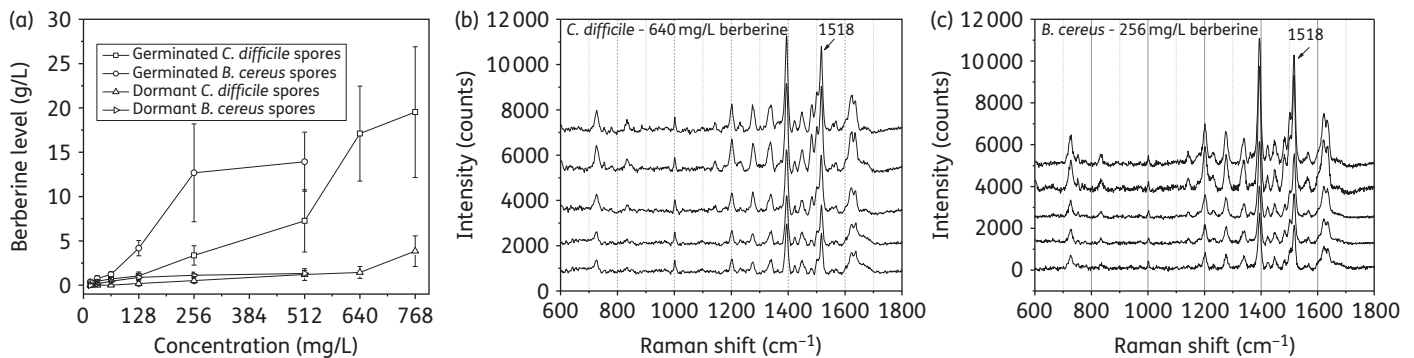

Figure 3. Berberine levels accumulated by single germinated and dormant *C. difficile* and *B. cereus* spores as determined by LTRS. (a) Average berberine levels of ∼50 individual germinated and dormant spores incubated with various concentrations of berberine. *C. difficile* and *B. cereus* spores were germinated for 60 min in either BHIS or LB medium plus germinants as described in the Materials and methods. Dormant *C. difficile* and *B. cereus* spores were incubated in 10 mM Tris–HCl (pH 7.4)–150 mM NaCl and 25 mM K-HEPES (pH 7.4) buffer, respectively. (b, c) Raman spectra of five individual spores germinated as described in the Materials and methods with the appropriate MIC of berberine. The arrows indicate the berberine-specific Raman band at 1518 cm^{-1} used to determine berberine levels.
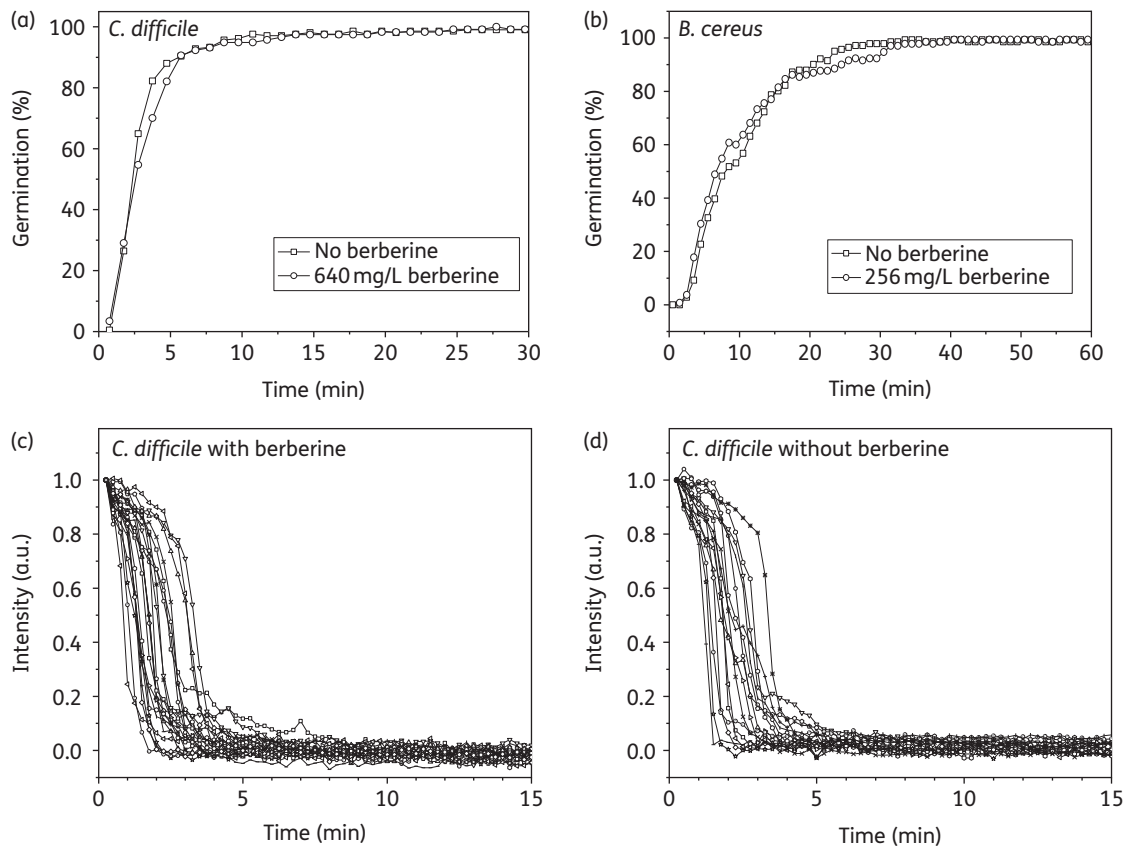

Figure 4. Germination of multiple individual *C. difficile* and *B. cereus* spores with or without berberine addition. (a) *C. difficile* spores were germinated in BHIS medium plus germinants as described in the Materials and methods without or with 640 mg/L berberine. (b) *B. cereus* spores were germinated in LB medium plus germinant as described in the Materials and methods without or with 256 mg/L berberine. The curves shown in (a) and (b) are averages of data for >200 individual spores. (c, d) DIC image intensities of ∼20 individual *C. difficile* spores germinating as described above with (c) or without (d) 640 mg/L berberine.

concentrations inside these two types of germinating spores are also different is not clear. In order to clarify this point, *C. difficile* and *B. cereus* spores were incubated, respectively, in BHIS medium plus germinants and in LB medium plus L-valine as well as with various berberine concentrations, and LTRS was used to determine berberine levels. For germinated *C. difficile* and *B. cereus* spores, when the berberine concentrations were below the MIC value, as berberine concentrations increased the berberine levels inside the germinated spores also increased (Figure 3). At the berberine MIC, the berberine levels in single germinated *C. difficile* and *B. cereus* spores were 17.1 ± 5.4 and 12.7 ± 5.5 g/L, respectively, and the ratios of berberine concentrations inside to outside spores were, respectively, 27 and 50 (Figure 3). When berberine concentrations were increased above the MIC values of the two kinds of spores, the berberine levels inside individual spores did not change much (Figure 3). It was also notable in these LTRS measurements at the berberine MIC that berberine levels inside individual germinated

spores of these two species showed significant heterogeneity (Figure 3). In contrast to the high berberine levels in germinated spores at the berberine MIC, for single dormant *C. difficile* and *B. cereus* spores at the berberine MIC, the berberine levels in spores were 1.2 ± 0.7 and 1.1 ± 0.1 g/L, respectively, and the ratios of berberine concentrations inside to outside spores were 2 and 4, respectively (Figure 3).

During germination, berberine's rapid uptake into spores began late in rapid CaDPA release and ended after completion of cortex lysis

As noted above, a large amount of berberine was taken up into germinated *C. difficile* and *B. cereus* spores and inhibited spore outgrowth. An obvious question is when precisely is berberine taken up during spore germination? Before addressing this question, we examined whether berberine might actually affect spore
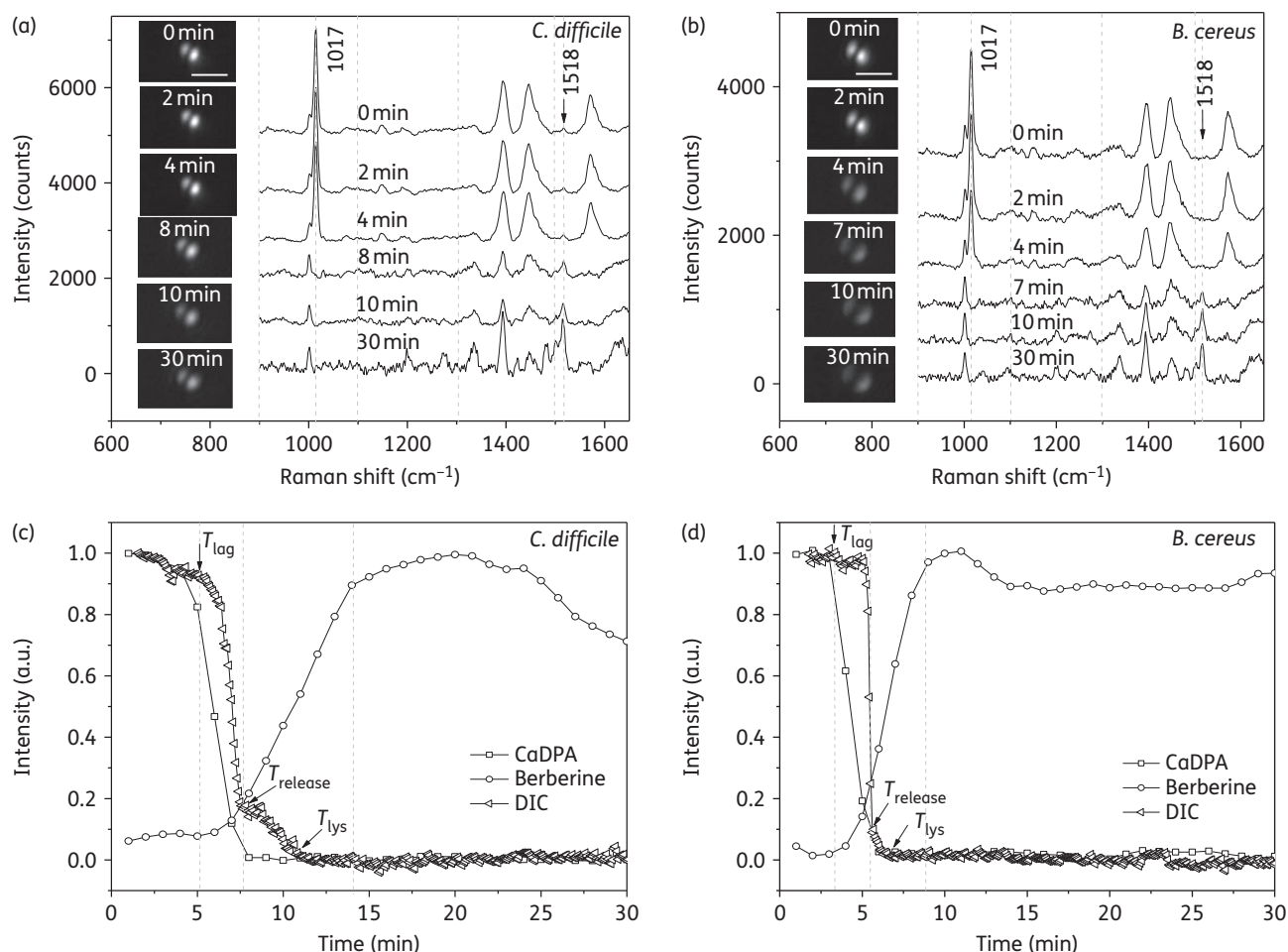

Figure 5. Time-lapse Raman spectra and DIC images of single optically trapped *C. difficile* and *B. cereus* spores during germination, and kinetics of changes in CaDPA levels and DIC image intensities. (a) DIC images and time-lapse Raman spectra of an individual *C. difficile* spore germinating in BHIS medium plus germinants as described in the Materials and methods with 256 mg/L berberine. (b) DIC images and time-lapse Raman spectra of an individual *B. cereus* spore germinating in LB medium plus L-valine, as described in the Materials and methods, with 64 mg/L berberine. The scale bar is 2.5 μm in the DIC images in panels (a) and (b). (c, d) Intensities of CaDPA- and berberine-specific Raman bands at 1017 and 1518 cm^{-1}, and intensities of DIC images of single trapped *C. difficile* and *B. cereus* spores as a function of germination time. The arrows indicate T_{lag}, $T_{release}$ and T_{lys}. CaDPA band intensities at 1017 cm^{-1} were normalized to the initial values at the first time of measurement, and berberine band intensities at 1518 cm^{-1} were normalized to the maximum intensity value, as described in the Materials and methods.

germination by monitoring germination of multiple individual spores with or without berberine addition using DIC microscopy (Figure 4).[21] The results of this experiment showed that, even at the berberine MIC, berberine did not affect any step in *C. difficile* and *B. cereus* spore germination. Furthermore, multiple average kinetic parameters of the germination of a large number of individual *C. difficile* and *B. cereus* spores with and without berberine were identical (Table S1, available as Supplementary data at *JAC* Online). Thus, berberine clearly has no effects on germination of *C. difficile* or *B. cereus* spores.

Since berberine had no effects on spore germination itself, we used LTRS and DIC microscopy to simultaneously monitor berberine uptake, CaDPA release and spore cortex hydrolysis, during the germination of multiple individual spores (Figure 5 and Figure S1). Notably, an individual *C. difficile* spore and an individual *B. cereus* spore gave similar kinetics of berberine uptake relative to other events in spore germination; similar kinetics for berberine uptake relative to CaDPA release and cortex lysis were also observed for a number of other individual germinating *C. difficile* and *B. cereus* spores (Figure S1). In the early minutes of spore germination, only small amounts of berberine were taken up, and this amount did not change until T_{lag}, when spores began to rapidly release CaDPA and to slowly accumulate more berberine. However, when rapid CaDPA release was nearing completion at $T_{release}$, berberine uptake became more rapid but then stopped shortly after T_{lysis}, when spore cortex hydrolysis was completed.

Discussion

Previous work has shown that berberine inhibits growth of many Gram-negative and Gram-positive bacteria,[11,12] and the mechanisms of berberine action include binding to nucleic acids as well as to the protein FtsZ and blocking cell division.[13–15] In the current work, we have confirmed that berberine inhibits *C. difficile* and *B. cereus* cell growth from spores, including outgrowth of germinated spores. However, berberine gave no inhibition of spore germination, presumably because neither nucleic acids nor cell division is required for spore germination. Thus, it seems likely that berberine can be used with spore germinants to kill spores immediately after spores germinate and minimize potential hazards of germinated and outgrowing spores. A low level of berberine (∼2-fold higher than berberine concentrations in the surrounding medium) was accumulated in dormant *C. difficile* and *B. cereus* spores prior to CaDPA release. However, it is likely that most of the berberine accumulated had penetrated the dormant spores' exosporium and was bound to the spores' coats, but it is very unlikely that this berberine had penetrated into the spore core, where nucleic acids are located, prior to CaDPA release and spore cortex peptidoglycan hydrolysis.

Usually, some concentration of antibiotics is required to inhibit cell growth and the minimum concentration needed to obtain complete growth inhibition is defined as an antibiotic's MIC value. However, under the condition of antibiotic MICs the actual amounts that penetrate into single cells are less well described, perhaps due to the lack of proper measurement techniques. Here, LTRS was used to measure the levels of the antibiotic berberine inside individual germinated spores. The results showed that, although the berberine MIC for *C. difficile* spores was ∼2.5-fold higher than that for *B. cereus* spores, berberine levels inside germinated *C. difficile* spores incubated at berberine's MIC were similar

to those for *B. cereus* spores (17.1 ± 5.4 versus 12.7 ± 5.5 g/L) (Figure 3), suggesting that the berberine levels needed inside germinated spores to inhibit bacterial spore growth may be similar for spores of different Gram-positive spore formers. Notably, the berberine levels in germinated spores showed significant heterogeneity (Figure 3). The reasons for this are not clear, but may be related to heterogeneity in levels of proteins involved in berberine import or export, or even significant variation in spore size.

It should be mentioned that in some of this work (Figures 3 and 5), LTRS measurements of berberine levels in *C. difficile* spores during germination were not carried out anaerobically, and the exposure of these germinating spores to oxygen might have interfered with ATP production and thus decreased the activity of ATP-dependent enzymes that excrete berberine. Therefore, the measured berberine accumulation in *C. difficile* spores might be higher than if germination and outgrowth were carried out anaerobically. However, as shown in the experiment in Figure 1, at the berberine MIC value the *C. difficile* spores did undergo germination but their outgrowth was inhibited by the berberine that entered the spores immediately after rapid CaDPA release. It is most likely that ATP production and usage in the germinating spores prior to the spore's outgrowth had not been activated.[1] Therefore, the value of berberine accumulation in *C. difficile* germinating/outgrowing spores aerobically could well be similar to what might be accumulated if this germination experiment was carried out anaerobically.

The uptake of berberine into growing bacterial cells has been measured previously using fluorescence spectroscopy,[27,28] but these measurements were generally restricted to population measurements using relative fluorescence intensity. While the fluorescence of berberine could be influenced by the intrinsic fluorescence of bacterial cells and the surrounding medium, Raman spectroscopy gives molecule-specific measurement of the berberine level. Here, LTRS was able not only to measure berberine uptake for single cells, but also to accurately quantify berberine content per cell. In general, as long as an antibiotic has specific and significant Raman spectral peaks and is accumulated inside cells to a significant level, LTRS will be useful to monitor this antibiotic's uptake and its levels inside cells.

Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1 Setlow P, Johnson EA. Spores and their significance. In: Doyle M, Beuchat L, eds. *Food Microbiology: Fundamentals and Frontiers*. Washington, DC: ASM Press, 2012; 45–79.

2 Kelly CP, LaMont JT. *Clostridium difficile*—more difficult than ever. *New Engl J Med* 2008; **359**: 1932–40.

3 Kotiranta A, Lounatmaa K, Haapasalo M. Epidemiology and pathogenesis of *Bacillus cereus* infections. *Microbes Infect* 2000; **2**: 189–98.

4 Ali S, Moore G, Wilson APR. Spread and persistence of *Clostridium difficile* spores during and after cleaning with sporicidal disinfectants. *J Hosp Infect* 2011; **79**: 97–8.

5 Baines SD, O'Connor R, Saxton K *et al.* Activity of vancomycin against epidemic *Clostridium difficile* strains in a human gut model. *J Antimicrob Chemother* 2009; **63**: 520–5.

6 Roberts TA, Baird-Parker AC, Tompkin RB. *Characteristics of Microbial Pathogens*. London: Blackie Academic and Professional, 1996.

7 Setlow P. Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J Appl Microbiol* 2006; **101**: 514–25.

8 Cowan MM. Plant products as antimicrobial agents. *Clin Microbiol Rev* 1999; **12**: 564–82.

9 Zuo F, Nakamura N, Akao T *et al.* Pharmacokinetics of berberine and its main metabolites in conventional and pseudo germ-free rats determined by liquid chromatography/ion trap mass spectrometry. *Drug Metab Dispos* 2006; **34**: 2064–72.

10 Taylor CT, Winter DC, Skelly MM *et al.* Berberine inhibits ion transport in human colonic epithelia. *Eur J Pharmacol* 1999; **368**: 111–8.

11 Yu HH, Kim KJ, Cha JD *et al.* Antimicrobial activity of berberine alone and in combination with ampicillin or oxacillin against methicillin-resistant *Staphylococcus aureus*. *J Med Food* 2005; **8**: 454–61.

12 Hwang BY, Roberts SK, Chadwick LR *et al.* Antimicrobial constituents from goldenseal (the rhizomes of *Hydrastis canadensis*) against selected oral pathogens. *Planta Med* 2003; **69**: 623–7.

13 Domadia PN, Bhunia A, Sivaraman J *et al.* Berberine targets assembly of *Escherichia coli* cell division protein FtsZ. *Biochemistry* 2008; **47**: 3225–34.

14 Boberek JM, Stach J, Good L. Genetic evidence for inhibition of bacterial division protein FtsZ by berberine. *PLoS One* 2010; **5**: e13745.

15 Bhadra K, Maiti M, Kumar GS. Berberine-DNA complexation: new insights into the cooperative binding and energetic aspects. *Biochim Biophys Acta* 2008; **1780**: 1054–61.

16 Serafim TL, Oliveira PJ, Sardao VA *et al.* Different concentrations of berberine result in distinct cellular localization patterns and cell cycle effects in a melanoma cell line. *Cancer Chemother Pharmacol* 2008; **61**: 1007–18.

17 Mantena SK, Sharma SD, Katiyar SK. Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. *Mol Cancer Ther* 2006; **5**: 296–308.

18 Huang SS, Chen D, Pelczar PL, Vepachedu VR, Setlow P, Li YQ. Levels of Ca^{2+}-dipicolinic acid in individual *Bacillus* spores determined using microfluidic Raman tweezers. *J Bacteriol* 2007; **189**: 4681–7.

19 Kong L, Zhang P, Wang G *et al.* Characterization of bacterial spore germination using phase-contrast and fluorescence microscopy, Raman spectroscopy and optical tweezers. *Nat Protoc* 2011; **6**: 625–39.

20 Wang S, Setlow P, Li Y-q. Slow leakage of Ca-dipicolinic acid from individual *Bacillus* spores during initiation of spore germination. *J Bacteriol* 2015; **197**: 1095–103.

21 Zhang P, Kong L, Wang G *et al.* Combination of Raman tweezers and quantitative differential interference contrast microscopy for measurement of dynamics and heterogeneity during the germination of individual bacterial spores. *J Biomed Opt* 2010; **15**: 056010–9.

22 Nerandzic MM, Donskey CJ. Activate to eradicate: inhibition of *Clostridium difficile* spore outgrowth by the synergistic effects of osmotic activation and nisin. *PLoS One* 2013; **8**: e54740.

23 Adams CM, Eckenroth BE, Putnam EE *et al.* Structural and functional analysis of the CspB protease required for *Clostridium* spore germination. *PLoS Pathog* 2013; **9**: e1003165.

24 Yi X, Setlow P. Studies of the commitment step in the germination of spores of *Bacillus* species. *J Bacteriol* 2010; **192**: 3424–33.

25 Wang S, Shen A, Setlow P *et al.* Characterization of the dynamic germination of individual *Clostridium difficile* spores using Raman spectroscopy and differential interference contrast microscopy. *J Bacteriol* 2015; **197**: 2361–73.

26 Olguín-Araneda V, Banawas S, Sarker MR *et al.* Recent advances in germination of *Clostridium* spores. *Res Microbiol* 2015; **166**: 236–43.

27 Ball AR, Casadei G, Samosorn S *et al.* Conjugating berberine to a multidrug efflux pump inhibitor creates an effective antimicrobial. *ACS Chem Biol* 2006; **1**: 594–600.

28 Samosorn S, Tanwirat B, Muhamad N *et al.* Antibacterial activity of berberine-NorA pump inhibitor hybrids with a methylene ether linking group. *Bioorg Med Chem* 2009; **17**: 3866–72.