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 (Tlag) when rapid release of the spore core’s large depot of the 1:1 chelate of Ca2+ with dipicolinic acid began, and the level of berberine taken up was maximal shortly after spore cortex lysis was completed (Tlysis).

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 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. 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. For example, B. cereus foodborne illnesses occur primarily due to survival of the bacterial spores when food is improperly cooked, as cooking temperatures ≤100°C allow some B. cereus spores to survive. 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. 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. 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. This compound has been used as a broad-spectrum antimicrobial agent for many years in traditional Chinese, Native American and Western medicine. Berberine has been shown to exhibit tumoricidal and anti-inflammatory activities. and MRSA. Berberine's antibacterial activity has been ascribed to effects on cell membranes, interactions with DNA and inhibition of cell division. In addition, berberine has been shown to exhibit tumoricidal and anti-inflammatory activities. 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. 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\(^{-1}\); (iii) since CaDPA release is a major event in spore germination, measurement of CaDPA release and berberine uptake kinetics simultaneously will allow place-ment 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; and (v) single-cell measurements allow characterization of cellular heterogeneity in levels and uptake kinetics of berberine among a population of germinating spores. 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 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. B. cereus spores were prepared and purified as described previously. 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 (10^6 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. 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. 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. 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 spectrophotograph. 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. 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. 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 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, 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 \( T_{\text{release}} \). 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 \( T_{\text{release}} \). In addition to \( T_{\text{release}} \), the parameters \( T_{\text{lag}} \), the time when rapid CaDPA release began, and \( T_{\text{ergy}} \), 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. 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.

### 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). 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. 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](http://jac.oxfordjournals.org/)

**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

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**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, \textit{C. difficile} and \textit{B. cereus} spores were incubated, respectively, in BHIS medium plus germinants and in LB medium plus \textit{l}-valine as well as with various berberine concentrations, and LTRS was used to determine berberine levels. For germinated \textit{C. difficile} and \textit{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 \textit{C. difficile} and \textit{B. cereus} spores were $17.1 \pm 5.4$ and $12.7 \pm 5.5$ 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 \textit{C. difficile} and \textit{B. cereus} spores at the berberine MIC, the berberine levels in spores were $1.2 \pm 0.7$ and $1.1 \pm 0.1$ 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 \textit{C. difficile} and \textit{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

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**Figure 5.** Time-lapse Raman spectra and DIC images of single optically trapped \textit{C. difficile} and \textit{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 \textit{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 \textit{B. cereus} spore germinating in LB medium plus \textit{l}-valine, as described in the Materials and methods, with 64 mg/L berberine. The scale bar is 2.5 mm 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 \textit{C. difficile} and \textit{B. cereus} spores as a function of germination time. The arrows indicate \(T_{\text{lag}}\), \(T_{\text{release}}\), and \(T_{\text{lys}}\). 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). 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 Tlag, when spores began to rapidly release CaDPA and to slowly accumulate more berberine. However, when rapid CaDPA release was nearing completion at Trelease, berberine uptake became more rapid but then stopped shortly after Tsys, when spore cortex hydrolysis was completed.

Discussion

Previous work has shown that berberine inhibits growth of many Gram-negative and Gram-positive bacteria, and the mechanisms of berberine action include binding to nucleic acids as well as to the protein FtsZ and blocking cell division. 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 spore 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. 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, 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.

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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


