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The hydration number *n* of calcium dipicolinate trihydrate, CaDP·nH_2O, and its effect on the IR spectra of sporulated *Bacillus* bacteria

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ABSTRACT

Previous results have shown a unique "quartet" of peaks in the infrared spectra of the sporulated phase of *Bacillus* bacteria, the four peaks being observed reproducibly for many different species of *Bacillus* endospores. We consistently observe bands at 766, 725, 701, and 659 cm^{-1} with the same relative amplitudes, as well as other spore peaks at 1441, 1277, and 1015 cm^{-1}. We have suggested that the peaks arise from calcium dipicolinate, not the conjugate acid. In this paper we conduct a theoretical and experimental study to show that the IR peaks not only arise from the calcium dipicolinate, a known spore component, but specifically the trihydrate salt, CaDP·$3H_2O$. This is shown by calculating the *absolute* IR frequencies and intensities of the lone dipicolinate dianion, the calcium salt, as well as the mono-, di- and tri-hydrate salts of calcium dipicolinate. The quartet peaks arise from the crystalline trihydrate salt as verified both experimentally and using quantum chemistry methods. When the calculated intensities are not normalized, only the trihydrate spectrum shows low frequency modes (below 1000 cm^{-1}, including the quartet) having intensities comparable to those of the pyridine ring. The vibrational modes in this region are associated with many internal coordinate motions including contributions from the Ca^{2+} counterion and the three waters including Ca–O–H bends, H_2O–Ca–O torsions and O–C–O bends.

1. Introduction

There exists a continuing need for methods to detect, identify and quantify an increasing number of threats to the general population including from explosives, toxic chemicals and biological threat species. While no one method or technology can detect all possible weapons of mass destruction (WMD), many continue to be investigated as to their specialized efficacies. The threat posed by biological agents is significant as both viruses and bacteria can infect large numbers of people with relatively small amounts of material [1–4]. The ability to accurately and rapidly detect species such as *Bacillus anthracis* bacteria is thus paramount. Such Gram-positive bacteria have the ability to sporulate, i.e. form endospores that can survive in environments lacking in growth media and that are otherwise chemically inhospitable, e.g. heat, strong pH, etc. The spores can survive in such a dormant state for extended periods, up to months or even years.

One of the techniques used to detect bacteria, including in the sporulated states, is broadband Fourier transform infrared (FTIR) spectroscopy [5–9]. FTIR has been used to investigate bacteria for several years, and has been shown capable of reasonably sensitive detection with the ability to discriminate between different species, and easily discriminate between biological species and sundry background materials. The group of Naumann and coworkers established early on the utility of IR to discriminate and differentiate between similar *Bacillus* species [10–13]. Naumann, Helm and Schultz also successfully conducted some investigations as to the nature of the chemical moieties giving rise to the IR bands [13–15]. It is well known, for example, that the amide I and amide II protein peaks are at 1657 and 1541 cm^{-1}, respectively, as well as the broad water O–H bands in the 3200–3500 cm^{-1} domain. Other known bands include the broad peak near 1250 cm^{-1} ascribed to the phosphate moiety in phospholipids. Previous work from our group showed that both photoacoustic infrared spectroscopy [16] as well as transmissive infrared spectroscopy [17] can be used to not only classify different species of bacteria, but when combined with chemometrics both methods can readily distinguish cells in the vegetative from those in the sporulated state [18].

In two recent papers, we have made advances correlating chemical species with certain IR bands. For vegetative cells [19], we consistently observed a peak near 1739 cm^{-1} that we ascribed to phospholipids, likely one or both of the species phosphatidylethanolamine (PE) or phosphatidyl glycerol (PG). The presence of such species, likely PG, was confirmed using a Bligh-Dyer extraction and mass spectrometry. It was also observed that this lipid peak could be used as a telltale marker for either vegeta-

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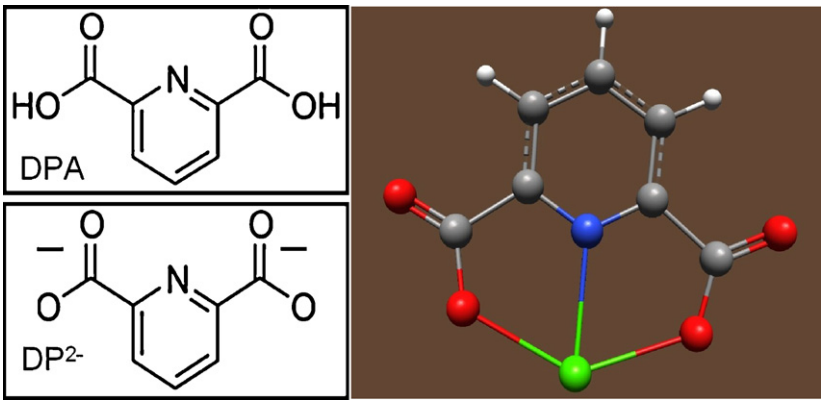

Fig. 1. Chemical structure of dipicolinic acid (DPA) and dipicolinate anion (DP^{2-}). At right is shown the structure of the anion with a calcium counterion assuming a planar C_{2v} geometry.

tive cells or vegetative cell debris amongst spore spectra, as small traces of the ~1735 cm^{-1} lipid peak would appear as a small shoulder on the 1657 cm^{-1} amide I band. This is likely associated with the difficulty of obtaining sporulated cells in a 100% pure state. Conversely, for the sporulated cells [20], while the band at 1735 was either diminished or absent, there was consistently a set of four peaks that was observed in the IR spectra of all sporulated bacteria. We have routinely observed this longwave infrared (LWIR) "quartet" of peaks at 766, 725, 701 and 659 cm^{-1}. The bands are very reproducible in their frequencies and relative intensities, and are observed in the endospore spectra but not for the vegetative cells. Several groups, including those of Myrick et al. [21], Eversole and co-workers [22], Samuels et al. [23] as well as Goodacre et al. [24], have indicated that such bands may be associated with either dipicolinic acid (DPA) or the conjugate salt calcium dipicolinate (CaDP). These chemical substances are known to be at elevated concentrations in sporulated cells; their chemical structures are presented in Fig. 1. The present work is primarily a theoretical effort to better verify the chemical nature (acid/base) of the substance as well as its local environment in terms of counterions and waters of hydration that give rise to the characteristic spore signatures by modeling the observed infrared spectra.

Our previous studies have shown that the infrared spectra of different *Bacillus* species are very similar, yet there exist small but reproducible differences in the spectra that allow discrimination versus background materials and even versus similar species down to the strain level [16–18]. We have also shown that the analytical reproducibility is high, including for the described quartet of peaks. However, there does exist some variation in the batch-to-batch results, even though the samples are from the same strain and cultured and harvested under identical conditions. Thus, in order to understand general aspects of the chemical composition of such bacteria, we have conducted expansive studies using different species. Specifically, eight different *Bacillus* species were chosen for these studies: (1) *Bacillus cereus* ATCC® 14579, (2) *Bacillus thuringiensis* subsp. *kurstaki* ATCC® 33680, (3) *Bacillus licheniformis* ATCC® 14580, (4) *Bacillus megaterium* ATCC® 14581, (5) *Bacillus thuringiensis* subsp. *israelensis* ATCC® 35646 and 39152, (6) *Bacillus mycoides* ATCC® 21929, (7) *Bacillus subtilis* ATCC® 49760, and (8) *B. subtilis* ATCC® 23857.

2. Experimental

The experimental methods used to study the bacteria have been previously described [16–20]. Briefly, the bacterial samples are cultured and deposited from solution onto a wedged ZnSe window and allowed to dry. The spectra were recorded using either of two different FTIR spectrometers, one of which is a vacuum bench that removes carbon dioxide and water vapor interferents, and improved intensity stability. Both the sample spectrum and a blank of the same empty window spectrum are recorded and ratioed to one another to generate a $\log_{10}$ absorbance file. To minimize spectral variability due to different batches, a composite or average spectrum was generated for each species. For the spores, this was done by repeating the culturing process at least five different times on different days. From each culture, the analytical procedure was repeated at least 3 times. Hence, typically 15 separate spectra are used to create each composite. A similar procedure was used for the vegetative cells, but in those cases only two or three cultures were generated as the reproducibility of the procedure is greater.

2.1. Infrared spectroscopy

Both a Bruker IFS 66v/S vacuum bench and a Bruker Tensor 37 purged infrared spectrometer were used for these studies as previously documented [25,26]. The bacteria IR spectra were recorded at 4.0 cm^{-1} resolution using standard mid-infrared components with the samples deposited on ZnSe substrates. The domain was limited by the ZnSe, but most of the spectral features are found in the 1800–500 cm^{-1} range. Each plotted spore spectrum typically represents 15 or more spectra as described above. For the DPA and CaDP samples KBr pellets were pressed in the usual fashion and these data were recorded on a Bruker Tensor 27 spectrometer which was used at 2.0 cm^{-1} since some of the spectral features, e.g. the 1277 cm^{-1} band, appeared quite sharp. For all experimental spectra the absorbance spectra have been scaled from 0.0 to 2.0 as the amount of material is not quantitative.

2.2. Bacteria preparation

The methods used to grow the bacteria as well as the media used to culture them have been detailed [19,20]. The American Type Culture Collection was the source of the bacterial stocks mentioned above. In terms of growth, colony counts showed cultures containing approximately 3×10^8 CFU/mL (CFU = colony forming units). Spores were cultured by incubating for several days on the appropriate medium until examination under a microscope (1000× magnification) indicated ≥95% spores. The spore suspensions were washed 4–5 times by centrifugation in sterile water to remove vegetative debris and media. Afterwards the spore pellet was resuspended in 2–5 mL of E-pure water to yield concentrations in the range 10^8–10^9 CFU/mL and stored at 4 °C until used. Bacterial spectra were typically inspected for abnormalities, instrument anomalies, and sample preparation anomalies such as culturing, storage or freezing irregularities.