High-Resolution Solid-State $^{13}$C Nuclear Magnetic Resonance of Bacterial Spores: Identification of the Alpha-Carbon Signal of Dipicolinic Acid

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Natural-abundance solid-state $^{13}$C nuclear magnetic resonance spectra were obtained for bacterial spores for the first time by using the technique of cross-polarization magic-angle-spinning nuclear magnetic resonance spectroscopy. A resonance at about 150 ppm, detectable in spore samples having a Mn content of less than 0.05%, was consistent with an identification as the α-carbon signal of calcium dipicolinate; this signal was missing from a spore sample treated with acid to release dipicolinate and from a spore coat preparation. Carbohydrate peaks were particularly intense in spores and coat preparations of Bacillus macerans. Signals ascribable to β-hydroxybutyrate were prominent in a B. cereus sample.

Dipicolinic acid (2,6-pyridinedicarboxylic acid; DPA) is a unique compound, present in large quantity (5 to 15%) in bacterial spores and only rarely elsewhere in nature (21). DPA appears to be involved in spore stability (10), spore germination (10), and heat resistance (3, 19, 20, 21). Present evidence suggests that DPA is primarily located in the spore core (15, 16), but its function and chemical state in the spore remain unknown. DPA has at various times been proposed to be tightly packed with the vital cell constituents (41), linked with protein (6, 21, 38), or intercalated with DNA (19). Evidence from UV (2), infrared (24), electron paramagnetic resonance (13, 42), and laser Raman spectroscopy (33) suggests it exists with Ca in some type of chelate, in a "dehydrated crystalline lattice" (13). It has not been possible to use the powerful tool of nuclear magnetic resonance (NMR) spectroscopy in exploring this problem because of line broadening due to the presence of large amounts of paramagnetic metals, especially Mn (5, 40), and strong nuclear magnetic dipolar interactions in solid samples. We attempted to circumvent these difficulties with low-Mn spores and the new techniques of cross-polarization (CP), magic-angle spinning (MAS), and high-intensity proton decoupling, already successfully applied to studies of intact bacterial cells (11). We present here the first $^{13}$C NMR spectra of bacterial spores, along with evidence that the α-carbon signal of DPA can be detected since it occurs in a window between 140 and 155 ppm in which no other spore components show strong resonances. This signal was present in all spore samples except those known to be lacking DPA, provided that the signal was not broadened beyond detectability by a Mn content in excess of 0.05%.

MATERIALS AND METHODS

Bacterial strains. Bacillus macerans B-70 and B-171 and B. megaterium ATCC 10778 (NRRL B-938) were obtained from the Northern Regional Research Center, Peoria, Ill. B. macerans 7X1 and B. coagulans 1491 were obtained from the National Food Processors Association, Washington, D.C. B. cereus TR-8 was obtained from R. S. Hanson, University of Wisconsin, Madison, and B. subtilis 168 was from R. Doi, University of California, Davis.

Preparation of spores. Spores of B. macerans were prepared as described previously (26). Spores of B. cereus TR-8 were prepared as described by Hanson et al. (10), and spores of B. megaterium ATCC 10778 were prepared as described previously (27); Lot 241-79 was purified in a two-phase system (Y [27]); Lot XIV, 169 was not. B. coagulans 1491 was grown on Thermoacidurans Agar (Difco Laboratories), adjusted to pH 7 with KOH, and supplemented with 1 μg of MnSO$_4$ per ml; spores were washed extensively and dried (26) under vacuum. B. subtilis 168 spores were grown on Schaeffer's sporulation medium 2XSG (17), purified (27), washed, and dried (26) under vacuum. All spores were stored at 5°C before use.

NMR spectroscopy. A JEOL GX-270 NMR spectrometer system equipped with a wide-bore Oxford solenoid and a Chemagnetics $^{13}$C ($^1$H) CP-MAS VT probe and associated high-power pulse amplifiers provided high-resolution solid spectra of natural-abundance $^{13}$C at a frequency of 67.9 MHz. All spore spectra were obtained at ambient temperature in 9.5-mm (outside diameter) Kel-F rotors having a volume of 0.3 ml, which were spun in the range of 3.1 to 3.4 kHz. Sensitivity seemed optimum with a contact time of 1 ms and a delay of 1 s between pulses. The 90° $^1$H flip time was set at 6 μs. The same $^1$H irradiation level was used for decoupling. An acceptable signal-to-noise ratio was usually obtained with 3,600 acquisitions, but whenever possible 21,600 pulses were collected. Data were acquired into 2,048-word blocks of memory which were zero-filled to 4,096 words before Fourier transformation. Exponential apodization, giving a line broadening of 50 Hz, was normally used. The TOSS (7) pulse sequence was frequently used to effectively suppress the spinning sidebands, although it was not required to view the DPA α-carbon region around 150 ppm. For Fig. 3C and 5B and C, the flipback sequence was used (39). It did not appear to provide any improvement in sensitivity and does not suppress the sidebands.
RESULTS AND DISCUSSION

The CP-MAS NMR spectrum of calcium DPA is shown in Fig. 1. Note the extremely prominent peak of the \( \alpha \)-carbon at 149.8 ppm (cf. references 6 and 30). This peak was detected in spore samples. For DPA (solid form), this signal occurs at 144.6 ppm. The signal observed in spores is thus consistent with that of the Ca chelate, which agrees with a recent laser Raman study (33). The spectrum of a spore sample of \( B. \) coagulans 1491 (Mn content, 0.022%) is shown in Fig. 2. Peak identifications were facilitated by recent publications (11, 31). The DPA \( \alpha \)-carbon resonance was readily visible at 150.3 ppm, displaced only about 0.5 ppm downfield from the model compound. Resonances from nitrogen base carbons of nucleic acids can be expected in this region (8). However, the concentration of these nitrogen bases is about an order of magnitude below that of DPA (21, 23) and hence would be undetectable under the conditions used here. The identification of the \( \alpha \)-carbon signal was confirmed as shown below. Figure 3A shows the spectrum of a spore preparation of \( B. \) macerans B-171 (0.016% Mn), showing a strong signal at 150.9 ppm. The same spores, treated briefly with 1.7 M HCl to release DPA (9, 25), did not show the signal (Fig. 3B). A spore coat preparation (Fig. 3C) of strain B-171 also lacked the \( \alpha \)-carbon signal at 150 ppm.

The DPA carboxyl carbon resonances at 169.7 and 171.7 ppm (Fig. 1) were completely obscured by the very large carbonyl signal centered at 173 (31) of the spores, which are known to contain over 65% protein (21), as well as several other sources of carboxyl (phosphoglyceric acid, fatty acid, muramic acid derivatives, etc.). The \( \beta \)- and \( \gamma \)-carbon resonances of CaDPA (Fig. 1) were obscured by protein components. The similarity of the spectrum of the

FIG. 1. Natural-abundance CP-MAS \(^{13}\)C NMR spectrum of CaDPA. Line assignments are given below. The scale is in parts per million downfield from tetramethylsilane as an external reference. Spectra were obtained as described in the text. The TOSS sequence did not completely suppress the spinning sidebands. The residual sidebands of the \( \alpha \)-carbon peak appear at 187.4 and 101.8 ppm. Carboxyl signals are at 171.2 and 169.7 ppm (two signals arise from the nonequivalence of all carboxyls in the solid state [37]), the \( \alpha \)-carbons are at 149.8 ppm, the \( \beta \)-carbons are at 128.1 ppm, and the \( \gamma \)-carbon is at 138.8 ppm (6, 30).

Nonetheless with respect to external tetramethylsilane were determined by assigning the signal from a Delrin rotor a shift of 88.0 ppm. Dry spores were gently packed by hand into the rotor cells.

Special spore treatments. Spores of \( B. \) macerans B-171 were suspended in 1.7 M HCl at 55°C for 15 min to release DPA (9, 25). After cooling, the spores were centrifuged in polypropylene centrifuge tubes, washed (5°C), and immediately dried in vacuo (26). Confirmation of DPA release was obtained by determining the \( A_{260.9} \) of the acid supernatant (18). Spore coats were prepared by dry rupturing (28) (150 mg of spores, 900 mg of NaCl, 3.4 g ball pestle) for 2 min in a vial (13 × 30 mm) and spun down after suspension in H\(_2\)O, followed by lysozyme (100 μg/ml) digestion (30 min, 37°C) in 0.1 M Tris hydrochloride (pH 8.0), washing, and drying under vacuum (26).

Analyses. Mn was analyzed by atomic absorption spectroscopy. DPA was assayed by the method of Janssen et al. (12); rough estimates were sometimes made by determining the \( A_{260.9} \) (18). The viability of dry spore preparations was determined, after activation (65°C, 20 min) of a water suspension, from the CFU on Plate Count Agar (Difco) and compared with Petroff-Hauser chamber counts. The viability of the \( B. \) macerans B-171 spore preparation was found to be 95%.

Chemicals. DPA was obtained from Aldrich Chemical Co. CaDPA · 3H\(_2\)O was prepared as described by Bailey et al. (2).
FIG. 3. CP-MAS 67.9-MHz $^{13}$C spectrum of (A) intact spores of *B. macerans* B-171 (Mn content, 0.0158%). Here, the $\alpha$-carbon signal of DPA is at 150.9 ppm. (B) Spores of *B. macerans* B-171 (A) treated briefly with strong HCl to release DPA and dried. Note the complete absence of signal at 150.9 ppm. (C) Lyophilized spore coats of *B. macerans* B-171. Note the absence of signal at 150.9 ppm. Spinning sidebands were not suppressed in this spectrum. The carbohydrate peaks near 70 ppm are unchanged from those of the untreated spores when the spinning sidebands are not suppressed. The sidebands of the carbonyl peak appear at 225 and 122 ppm. Peak heights obtained with different pulse sequences should not be compared.

FIG. 4. CP-MAS 67.9-MHz $^{13}$C spectrum of (A) intact spores of *B. macerans* 7X1 (Mn content, 0.0189%). The $\alpha$-carbon signal is prominent (150.5 ppm). (B) Intact spores of *B. macerans* B-70 (Mn content, 0.0413%); spinning sidebands are present in this spectrum. Note the weak signal of DPA at 150.5 ppm. Note also the many prominent carbohydrate peaks in the region of 60 to 80 ppm; these signals are particularly prominent only in the samples of *B. mace- ran* strains.
coats to that of the intact spores is consistent with their relatively large contribution to the dry weight of the spore (40 to 60% [21]). Two other B. macerans strains (Fig. 4A and B) also showed the α-carbon signal; the preparation of strain B70 (Mn content, 0.041%) showed only a weak α-carbon resonance (Fig. 4B), although its DPA content nearly equals that of strain B171. Since this signal does not appear to be appreciably broadened, one might speculate that the relative distribution of Mn and DPA found in these spores (Table 1) was different. All of the B. macerans spores (Fig. 3 and 4) and the coat preparation (Fig. 3C) showed a series of strong signals in the region of 60 to 80 ppm and at 100 ppm; these signals are characteristic of carbohydrate (31). B. macerans spores are known to possess unusual, thickly ridged coats (26) which might be a source of carbohydrate (a galactosamine–galactosamine-6-phosphate polymer is a major constituent of the coat in B. megaterium QM1551 [22]).

B. cereus is known to form large amounts of β-hydroxybutyrate (14, 21) granules, which are difficult to separate from B. cereus spores (14). Sharp peaks ascribable to β-hydroxybutyrate at 20.3, 67.9, and 169.2 ppm (11) were prominent in the spectrum of a preparation of B. cereus spores (Fig. 5A) and strongly suggest that this material is present in this preparation. Note the complete absence of the α-carbon signal of DPA at 150 ppm (Mn content, 0.16%). We were unable to detect the α-carbon signal in any spore sample with a Mn content of >0.05% (Table 1). The weak signals in the 155-ppm region seen in many of the spectra can be ascribed to the phenoxycarbon of tyrosine and the guanidino carbon of arginine (31), which are relatively abundant in spore protein (21, 41). Figure 5B shows the spectrum for a B. subtilis spore preparation with a Mn content of 0.31%; here, too, the α-carbon signal of DPA was not visible (Table 1; these spores also showed prominent carbohydrate peaks at 70 and 100 ppm). The spectra of two spore preparations of B. megaterium ATCC 10778, grown on a high-Mn medium, are shown in Fig. 5C and D. The preparation used for Fig. 5C was purified in a two-phase system effective in removing metal phosphate precipitates (21) and shows the major signals apparently associated with spore coats. Figure 5D shows a similar preparation made without treatment in the two-phase system; severe signal broadening occurred, possibly as a result of manganese phosphate precipitate on the spore surface. The fact that the main spectral features of the spore may be ascribed to the coat suggests that, when spores are well cleaned, the residual Mn is largely that chelated selectively to the DPA; Mn can replace Ca in DPA (34, 42) and, like DPA, appears to be concentrated in the spore core (35, 36). Only in the presence of a very high Mn concentration, some of which may be

**TABLE 1. Mn and DPA contents of spores**

<table>
<thead>
<tr>
<th>Strain</th>
<th>DPA %</th>
<th>Mn %</th>
<th>CP-MAS relative peak intensity at 150 ppm*</th>
<th>DPA/Mn molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. coagulans 1491</td>
<td>10.31</td>
<td>0.022</td>
<td>++++</td>
<td>154</td>
</tr>
<tr>
<td>B. macerans B-171</td>
<td>8.81</td>
<td>0.0158</td>
<td>++++</td>
<td>183</td>
</tr>
<tr>
<td>B. macerans 7X1</td>
<td>9.00</td>
<td>0.0189</td>
<td>++++</td>
<td>156</td>
</tr>
<tr>
<td>B. macerans B-70</td>
<td>8.55</td>
<td>0.0413</td>
<td>+</td>
<td>68</td>
</tr>
<tr>
<td>B. cereus TR-8wr</td>
<td>6.58</td>
<td>0.161</td>
<td>−</td>
<td>13</td>
</tr>
<tr>
<td>B. subtilis 168</td>
<td>10.45</td>
<td>0.313</td>
<td>−</td>
<td>11</td>
</tr>
<tr>
<td>B. megaterium ATCC 10778b</td>
<td>10.88</td>
<td>1.250</td>
<td>−</td>
<td>2.9</td>
</tr>
<tr>
<td>B. megaterium ATCC 10778c</td>
<td>11.44</td>
<td>2.28</td>
<td>−</td>
<td>1.6</td>
</tr>
</tbody>
</table>

* Estimates: −, no peak.

b Lot 241-79 (Fig. 3C).

c Lot, XIV, 169 (Fig. 5D).

surface located (purification with a two-phase system is known to remove metal phosphate precipitates from spore preparations [21]), were the major spectral features of the coat broadened excessively (Fig. 5D). The fact that the α-carbon appears to be so sensitive to Mn concentration also suggests preferential chelation of Mn by DPA. Since a DPA/Mn molar ratio of <68 appears to be associated with absence of the α-carbon signal (Table 1), one might presume that a packing of DPAs occurs around each Mn. This concentration of DPA molecules might be more consistent with a model in which calcium DPA is “tightly packed with the vital cell constituents…such that a variety of polar hydrogen bonding and aromatic ring stacking interactions occur” (41). A DPA-DNA complex (19) may also be consistent with the data. The sensitivity of the DPA signal to Mn may perhaps be exploited in future work on this topic.

The results presented here show that, with CP-MAS and low-Mn spores, NMR spectroscopy may be applied to 13C studies of bacterial spores. It appears necessary to work with very low-Mn spores, at least for DPA studies. Although Mn has long been thought to be required in high concentration for sporulation, this has not been found to be true in some recent studies (1, 29), and some spores containing low Mn concentrations have been reported previously (1, 21). Such low-Mn spores appear to arise as a result of genetic properties, method of preparation, or both. The low-Mn spores used in these experiments were obtained by growing on solid media with low Mn (B. coagulans) or massive excess Ca (B. macerans [26]). Contaminating Mn precipitates may sometimes be removed with aqueous polymer two-phase systems (21) or washing with dilute acid (21). In certain cases, Mn chelated to DPA might be removed by careful titration with acid (4). EDTA treatment has been applied to spores before 31P NMR spectroscopy (32).

DPA has at various times been proposed to be tightly packed with the vital cell constituents (41), linked with protein (6, 21, 38), or intercalated with DNA (19). It seems possible that, with the use of specifically labeled [13C]DPA-enriched spore samples to improve sensitivity, CP-MAS NMR may be able to contribute to the solutions of the above problems, as well as to other problems of spore biochemistry, e.g., carbohydrates prominent in certain spore coat preparations. Dipolar dephasing, combined with CP-MAS may be capable of separating the α-carbon peaks from any interfering protonated-carbon resonances in the same spectral region. 31P MAS NMR may also prove useful in high-resolution NMR studies directed toward the solid-phase phosphocarbohydrate and phospholipid components of spores and might provide spectra superior to those obtained with aqueous suspensions (32).
FIG. 5. CP-MAS 67.9-MHz $^{13}\text{C}$ spectrum of (A) intact spores of $B. \text{cereus}$ TR-8wr (mutant strain). This mutant strain (TR-8; originally lacking DPA) has apparently reverted and contains 6.58% DPA. The $\alpha$-carbon signal is not evident, presumably because of the high Mn content (0.161%). Note the strong peaks at 20.3, 67.9, and 169.2 ppm, clearly indicating the presence of $\beta$-hydroxybutyrate (9). (B) Intact spores of $B. \text{subtilis}$ 168 (Mn content, 0.313%). An $\alpha$-carbon signal is not evident. Note the prominent carbohydrate peaks at 70 and 100 ppm. (C) Intact spores of $B. \text{megaterium}$ ATCC 10778, Lot 241-79 (Mn content, 1.25%). (D) Intact spores of a different preparation (XIV, 169) of the same strain not purified in a two-phase system (Mn content, 2.28%). The spinning sidebands were not suppressed in spectra B, C, and D. A line-broadening factor of 200 rather than 50 Hz was used for B and D to reduce noise. The signal around 227 ppm in all of these spectra is the low-field spinning sideband of the carbonyl peak.
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LITERATURE CITED


