High-Resolution $^1$H NMR Spectroscopy of Metabolically Active Microorganisms Using Non-Destructive Magic Angle Spinning

The authors show that high-resolution $^1$H nuclear magnetic resonance (NMR) spectroscopy can be used to study biofilm metabolism under environmentally relevant conditions in a minimally invasive way.

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increased to ~30 Hz in biological objects. However, its sensitivity is superior to that of 1H PHORMAT, making it very attractive for studying small-sized actively metabolizing biological samples where the centrifugal force, proportional to the sample size, still is moderate (16, 21). For example, at a sample spinning rate of 40 Hz and using a sample rotor with an internal diameter of 6 mm, the maximum centrifugal force experienced by the sample only is 19 times the gravitational force, which is safe for studying a wide range of smaller, less sensitive organisms, organelles or tissues (17, 22). It has been demonstrated in a variety of excised organs and tissues that 1H slow-MAS PASS provides isotropic spectra with at least the same and sometimes better resolution than standard fast MAS (16, 17, 22).

In this article we will show that PASS can also be used successfully to study bacterial systems, even under conditions where the susceptibility broadenings are so large that the 1H NMR metabolite spectrum becomes virtually invisible. Hence, in order to avoid signal losses the spinning speed has to be large compared with $T_2$. In practice this means that for many biological applications minimum spinning speeds of 30–40 Hz are required (17).

All 1H NMR experiments were performed on a Chemagnetics (Varian, Inc., NMR Systems – Chemagnetics: Fort Collins, CO) 300 MHz Infinity spectrometer, corresponding to a proton Larmor frequency of 299,982 MHz. A standard Chemagnetics CP/MAS probe with a 6-mm i.d. pencil type spinner system was used. In order to be able to spin at low frequencies, the rotor was equipped with a flat drive tip (that is, it did not contain grooves, which normally are used to drive the rotor) and an airflow restriction was used in the driver channel. The spinning rate was controlled using a commercial Chemagnetics MAS speed controller. It was found that with this controller the frequency stability at 1 to 200 Hz is better than ±0.3 Hz, which appears to be sufficient for our experiments. In the experiments the durations of the π pulses used in the PASS sequences were 20–22 µs, and the DANTE sequences contained 4000–6000 pulses, spaced by 100 µs and with durations of 0.8–1.2 µs, corresponding to flip angles of 7–11 degrees.

**Results**

*Densely packed Shewanella MR-1 microbes. Shewanella oneidensis* MR-1 has been a subject of research for several years in our laboratory. These are dissimilatory metal-reducing bacteria that can catalyze the reduction of soluble metal ions including U(VI), Cr(VI) into insoluble forms, and have the potential for application in the remediation of contaminated surface and ground waters (29–31). Figure 2 illustrates the impact of 1H PASS on a sample of dense MR-1 cells. The cells were grown aerobically on agar plates for two days at room temperature on a minimal medium that is a slight modification of the medium used by Zachara et al. (32), with lactate as the carbon and energy source. Then the cells, which grew as a thick lawn, were harvested from the agar surface with a sterile plastic loop and inserted directly into the rotor. The observed metabolite spectrum is dominated by contributions from intracellular metabolites because the extracellular space is only a small fraction (~30%) of the total volume of the cell paste, and the concentrations of intracellular metabolites usually are much higher than those of the extracellular components. Figure 2a shows the spectrum obtained on a static sample. Although several peaks and shoulders can be observed, the lines are relatively broad. Hence, in the cells, relatively large susceptibility gradients exist, which originate from the boundaries of neighboring cells in this densely packed cell system. It was indeed observed that the lines become narrower when the cell concentration was decreased, confirming this conclusion. Figure 2b shows the stacked plot of PASS spectra obtained while spinning the sample at a speed of 40 Hz. Sixteen combinations of delay
times \(tm_1-tm_6\) were used, which makes it possible to separate the center band and 15 sideband spectra without spectral aliasing (16). In the figure, \(\omega_2\) is the chemical shift along the acquisition dimension with reference to TMS, and the parameter \(n\) denotes the center band \((n = 0)\) and the order of sideband spectra. Usually the center band spectrum offers the highest spectral quality, as it is less susceptible to pulse imperfections and instabilities of the spinning rate. Figure 2c displays the center band spectrum separately, showing a significant enhancement in the spectral resolution compared with the spectrum obtained in the static sample (Figure 2a). Based upon a proposed metabolite pathway of *Shewanella oneidensis* MR-1 (33), tentative spectral assignments of the peaks in Figure 2c were obtained by adding a few drops of a 200 mM solution of each possible metabolite into a sample with freshly collected MR-1 cells and by rapidly examining the intensity changes of the peaks observed in the resulting in 1H PASS spectrum. Tentative line assignments: 1 = Terminal methyl groups; 2 = methylene groups in carbohydrate chains; 3 = lactate methyl; 4 = alanine methyl; 5 = acetate methyl + CH\(_2\) protons in the unit of =CH-CH\(_2\); 6 = succinate methylene + isocitrate methylene; 7 = isocitrate methine + CH\(_3\) protons in the unit of =CH-CH\(_2\)-CH\(_2\); 8 = alanine methine; 9 = isocitrate methine (-CHOH-COOH); 10 = residual water intensity; and 11 = formate methine. The other intensities have not yet been assigned. Some of the assignments, such as lactate and alanine, were confirmed further by a two-dimensional double quantum filtered correlation spectroscopy (COSY) experiment. The other lines have not been assigned unambiguously, and this will be one of the topics of future research.

Figures 3a, b, and c show the 1H PASS center band spectra for agar plated MR-1 bacterial cells with incubation times of 2, 3, and 5 days, respectively, where the nutrient supply steadily decreases. It follows that, in response, the available metabolites are consumed and the cells become starved, and after 5 days only two intensities are observed, located at 1.2 and 2.0 ppm. The peak at 1.2 ppm could be due to methylene groups in fatty acid chains, perhaps arising from the extracellular polymer matrix made up of complex carbohydrates in which the cells are enrobed. The origin of the 2.0 ppm peak is most likely the methyl group of acetate, resulting from the oxidation of lactate. It has been shown that under anaerobic and microaerobic conditions acetate is excreted by MR-1 (33). Figure 3d shows the revival of the metabolic activities when a nutrient is added. Here the PASS spectrum is shown obtained 2.5 hours after adding 20 mm\(^3\) of a 200 mM solution of pyruvate to the 5-day incubated cell system. Almost all the metabolite peaks observed in Figure 3a were regenerated, albeit with different intensities (for example, the formate peak at 8.4 ppm is much more pronounced). More detailed and controlled experiments are planned to study the dynamics of bacterial metabolism in biofilms using the 1H PASS technique. Finally, it can be noted that the signal-to-noise ratio (S/N) in Figure 3b is slightly less than that in Figure 3a and the S/N is reduced significantly in Figure 3c, despite the equal amount of acquisitions. This trend can be explained by a decrease in the metabolite concentrations or a decrease in the metabolite \(T_2\)
values with increasing incubation times, the latter phenomenon resulting in signal losses during the first rotor period prior to the acquisition. The $T_2$ values of the metabolites in these samples are unknown, but we measured the $^1H$ $T_2$ of water using the Carr-Purcell-Meyboom-Gill (CPMG) method on static samples, and the resultant values are 10.0 ms (Figure 3a), 7.1 ms (Figure 3b) and 5.3 ms (Figure 3c). At a 40-Hz spinning speed this results in a factor of 10 signal attenuation in the water intensity observed after 5 days of incubation time, as compared with that obtained after 2 days. It is reasonable to expect that the $^1H$ $T_2$ values of metabolites follow the same trend as those of water, possibly explaining the observed reduction in S/N with increased incubation time.

Methods that combine PASS with relaxation measurements currently are under development in our laboratory so that $^1H$ $T_2$ of various metabolites in a biological sample can be measured separately.

**Shewanella putrefaciens CN-32 attached to solid surfaces.** In nature, many microbes typically are attached to solid surfaces such as sand and rock, and form biofilms. Often these solid materials have relatively large magnetic susceptibilities, resulting in an increased broadening of the NMR lines. To evaluate whether PASS can be used successfully for this type of system as well, we inserted Biosilon polystyrene spheres (Nunc A/S, Roskilde, Denmark, batch# 44047) and accusand (quartz) beads (Unimin Corp, Utica, IL, USA) with diameters of ~200 and ~60 µm, respectively, into a sample containing tryptic soy broth (TSB) growth medium (Fisher Scientific) with (30 g TSB powder/[liter water]) and *Shewanella putrefaciens* CN-32 cells (27). Before use, the accusand was cleaned using treatments of 0.25 M sodium hydroxide for 15 h, followed by 0.25 M nitric acid for 15 hours. The sand then was rinsed extensively with deionized water and was oven-dried at 105 °C. Before use the sand was autoclaved 20 min at 121 °C. Figure 4 shows the $^1H$ spectra obtained ~100 h (polystyrene beads) and 16 h (quartz beads) after sample preparation, during which time the microbes presumably have attached to the beads.

The NMR spectra shown in Figures 4a and 4d are obtained on static samples, and it is found that in the case of the sand beads the susceptibility broadening is so large that the metabolites become virtually unobservable with standard NMR (the line width of the water signal in this sample was about 800 Hz (~2.7 ppm)). This presumably is due to paramagnetic iron inclusions in the beads (34). Figures 4b and 4e show the PASS center band spectra, illustrating that PASS can be used for bacterial systems in the presence of solid materials as well. The susceptibility gradients arising from the quartz beads were so large that the MAS speed had to be increased from 40 to 100 Hz in order to avoid signal losses arising from the diffusion of the metabolites in these gradients during the first rotor period (this is similar to the case of water surrounding glass beads, where significant signals losses were observed in PASS experiments at spinning speeds of 50 Hz and below [19]). The spectrum in Figure 4b resembles that of the TSB growth medium itself embedded in the same type of polystyrene beads, which is shown in Figure 4c. Still significant differences are observed, such as increased intensities at 1.8 and 2.8 ppm in the spectrum shown in Figure 4b, arising from acetate and isocitrate, respectively, apparently pro-

![Figure 3. 40-Hz PASS $^1H$ center band spectra of densely-packed *Shewanella oneidensis* MR-1 bacteria grown on agar plates for (a) 2, (b) 3, and (c) 5 days, respectively. The spectrum shown under (d) was obtained 2.5 h after spiking the 5-days incubated microbes with 20 mM of a 200 mM solution of pyruvate. All the spectra were acquired using similar experimental parameters as those of the PASS spectra given in Figure 2 except that the number of scans used in (d) was 32. The water suppression in (d) is poor, which is primarily due to the fact that part of the added pyruvate solution leaked inside the sample rotor to places well beyond the homogeneous range of the RF coil.](image-url)
duced by the microbes. The spectrum in Figure 4e reveals only some of the intensities from the TSB and the spectrum is dominated by the lines arising from lipid chains at about 1.28 ppm, perhaps due to the lipid or polysaccharide matrix into which the microbes have embedded themselves.

**Conclusions**

It has been demonstrated that slow-MAS PASS makes it possible to study metabolic processes in living microbial systems in detail, even under conditions that in static samples the lines are so broad that they are difficult or impossible to distinguish using standard NMR techniques. MAS spinning rates as low as 40–100 Hz can be used, which makes the technique amenable to study small sized (a few mm) intact biofilms and other bacterial samples with no or little impact on their structural integrity. If larger samples need to be investigated and/or lower spinning speeds are required, the PHORMAT technique probably can be used, albeit at the cost of a reduced signal intensity and longer measuring times (16, 18, 21). However, its performance for bacterial systems still has not yet been evaluated. Finally, it should be noted that so far no attempt has been made to seal the rotor airtight or to inject nutrients during spinning. Efforts currently are underway to develop a rotating bioreactor that will make it possible to study bacterial systems under precisely controlled environmental conditions with slow-MAS techniques. This probe also will contain a gradient assembly that will be used for pulsed-field-gradient NMR experiments to separate the intra- and intercellular metabolites via their differences in diffusion coefficients, and for determining the spatial distribution of the metabolites in, for example, biofilms. If successful, we expect that with this approach metabolic processes can be studied in considerably more detail than was possible before, especially in samples directly obtained from nature, where often the NMR lines are broadened to a major extent due to its heterogeneous composition and the presence of sand grains and other solid materials.

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**References**


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