

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

The authors show that high-resolution ^1H 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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In vitro and in vivo ^1H nuclear magnetic resonance (NMR) spectroscopy increasingly is used for investigating biochemical processes in cells, tissues, animals, and humans (1–5). However, the ^1H spectrum often displays a poor resolution, which hampers a quantitative analysis of the spectra (4). It is known that in intact biological systems the susceptibility gradients, arising from variations in the isotropic bulk magnetic susceptibility near boundaries of inter- and intracellular structures, are the main sources for the observed line widths (6, 7). In principle, this broadening can be averaged to zero by the technique of magic angle spinning (MAS), in which the sample is rotated about an axis making an angle of $54^\circ 44'$ relative to the external magnetic field (8–10). High-resolution ^1H MAS spectroscopy at spinning rates ranging from a few kilohertz to more than 10 kHz has been used successfully to study metabolism in cells and excised tissues (3, 7, 11–15), offering a spectral resolution similar to that of solution NMR using cell extracts. These large spinning speeds are necessary to eliminate the spinning sidebands (SSBs) in the spectra (16), which occur at lower speeds and render the interpretation of the spectra difficult again. However, the large centrifugal forces associated with these spinning speeds destroy tissue and sometimes even cellular structures. Therefore the standard fast MAS approach is not viable for magnetic resonance spectroscopy of larger organisms or tissues that are subject to disruption by centrifugal forces, and methods are needed that yield high-resolution, SSB-free spectra at reduced MAS frequencies.

In our laboratory, we have modified two methods that eliminate the SSBs or separate them from the isotropic spectrum so that a SSB-free high-resolution isotropic spectrum is obtained, for ^1H NMR metabolite spectroscopy in biological samples (17–22). Both methods originally were developed especially for ^{13}C and ^{15}N solid-state NMR. One is an isotropic-anisotropic chemical shift 2D correlation experiment



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called ^1H PHORMAT (phase corrected magic angle turning) (18–21) and is evolved from the original PHORMAT experiment (23, 24). With ^1H PHORMAT, spinning rates as low as 1 Hz can be used (18), making the method viable for in vivo studies, as was recently demonstrated in a live mouse (21). However, a disadvantage of PHORMAT is a reduction in the NMR sensitivity and long measuring times (18–21). The second method is called ^1H PASS (phase adjusted spinning sidebands) (17), which is an extension of the original work reported by Antzutkin et al. (25) and Dixon (26, 27). With PASS, the minimum sample spinning rate needs to be

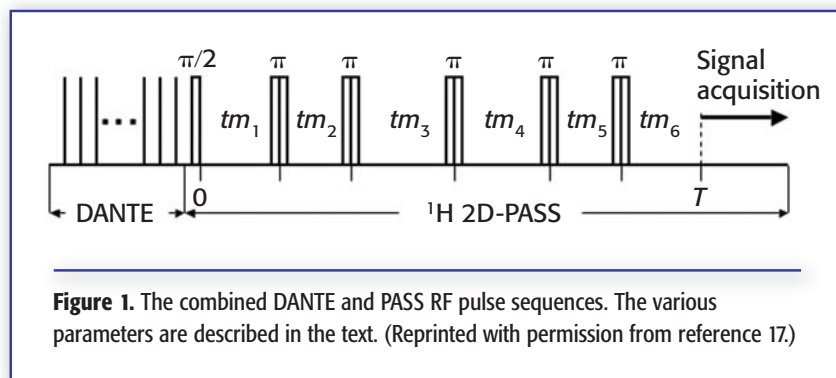


Figure 1. The combined DANTE and PASS RF pulse sequences. The various parameters are described in the text. (Reprinted with permission from reference 17.)

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 also can 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. Herein, we briefly review the ^1H PASS method and provide specific examples of applications recently carried out in our laboratory to evaluate the methodology in highly condensed bacterial cell pastes and bacterial cells grown as a biofilm attached to solid surfaces.

Experimental

PASS is a one-rotor-period constant evolution time 2D experiment, during which five π pulses are applied with time intervals tm_1 to tm_6 during the rotor period T (25). Figure 1 shows the PASS radio frequency pulse sequence that was used for the studies of biological sam-

ples (17). The major difference with the original sequence is the implementation of a water-suppressing DANTE (Delays Alternating with Nutations for Tailored Excitation) sequence (28), consisting of a train of equally spaced small tip angle hard pulses. In PASS the center band and the SSB spectra are separated by order. This is achieved by acquiring the signal after a series of PASS experiments with different values of the time intervals tm_1 to tm_6 . The theory of PASS sequence, its advantages and disadvantages, and the details of the implementation of the experiment are given in references (16, 17, 25). In PASS the signal acquisition starts after one rotor period T (Figure 1). During this period the signal decreases due to the spin-spin relaxation time T_2 associated with the isotropic line width. Hence, in order to avoid signal losses the spinning speed has to be large compared with $(T_2)^{-1}$. 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 ($\sim 30\%$) 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, ω_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 ^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 $=\text{CH}-\text{CH}_2$; 6 = succinate methylene + isocitrate methylene; 7 = isocitrate methine + CH_2 protons in the unit of $=\text{CH}-\text{CH}_2-\text{CH}=\text{}$; 8 = alanine methine; 9 = isocitrate methine ($-\text{CHOH}-\text{COOH}$); 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

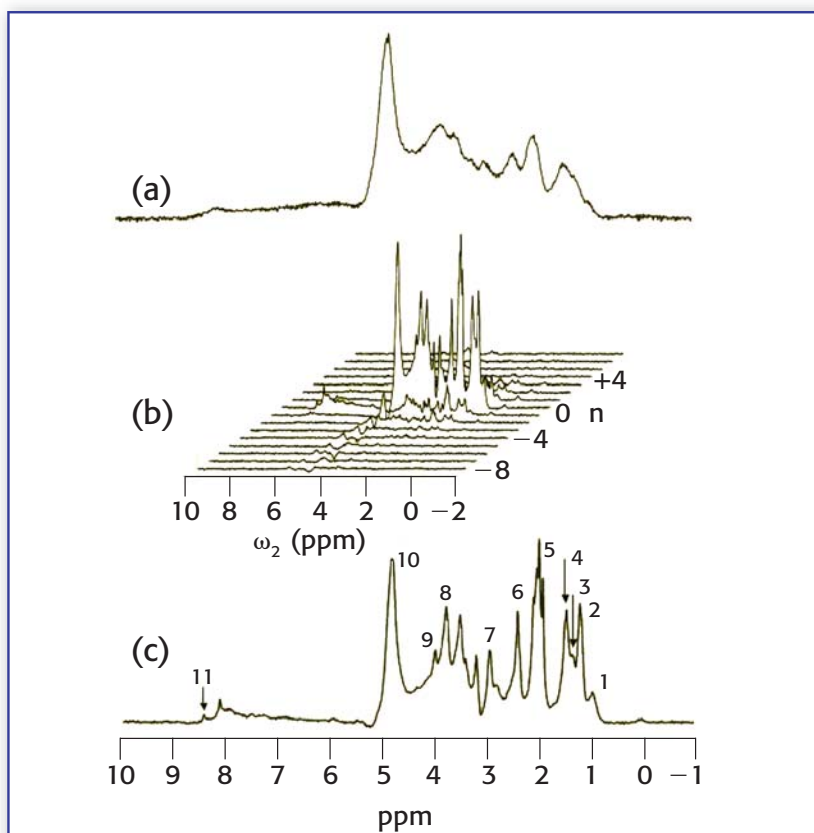


Figure 2. ^1H NMR metabolite spectra of densely-packed *Shewanella oneidensis* MR-1 bacteria (~ 150 mg) grown on agar plates for 2 days. (a) Spectrum obtained in a static sample; (b) stacked 2D plot obtained with ^1H PASS while spinning the sample at 40 Hz; (c) the center band spectrum of ^1H 2D PASS. The PASS spectrum was acquired using 16 evolution steps, tm_1 to tm_6 , (see Figure 1), allowing the separation of the center band ($n = 0$) and 15 SSB spectra. Each evolution step was acquired with 96 scans and a recycle delay time of 1 s. The carrier frequency for the DANTE sequence was centered at the center band of the water peak. The total measuring time was about 0.6 h.

could be due to methylene groups in methylene 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 $^\circ\text{C}$. Before use the sand was autoclaved 20 min at 121 $^\circ\text{C}$. 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 signal 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-

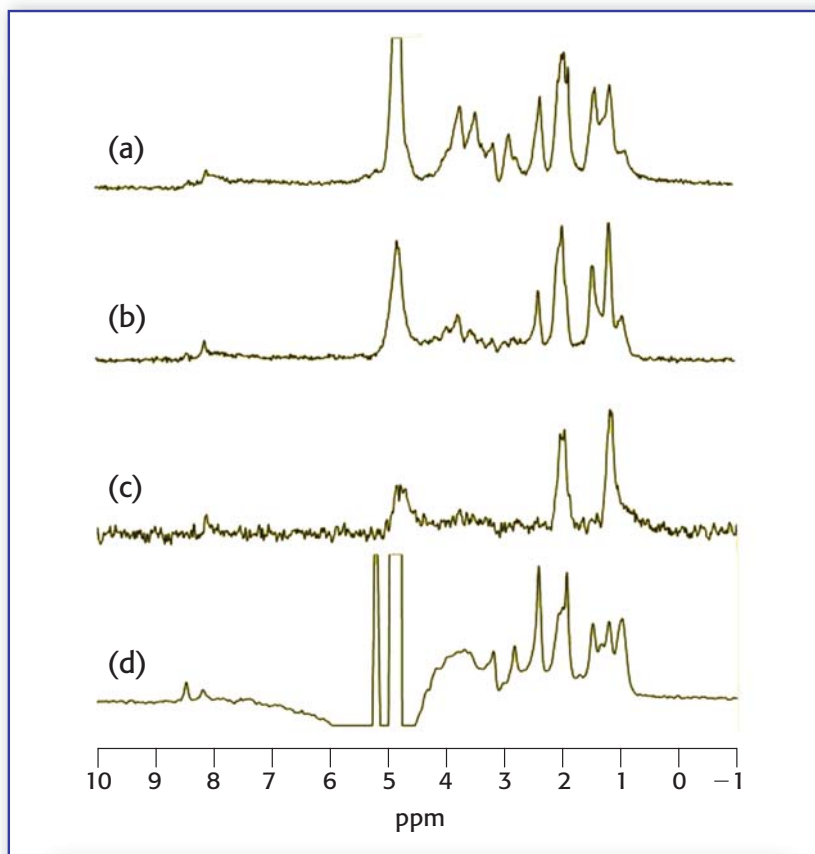


Figure 3. 40-Hz PASS ^1H center band spectra of densely-packed *Shewanella oneidensis* MR-1 bacteria grown on agar plates for (a) 2, (b) 3, and (c) 5 days, respectively. The spectrum shown under (d) was obtained 2.5 h after spiking the 5-days incubated microbes with 20 mm^3 of a 200 mM solution of pyruvate. 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.

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.

Acknowledgments

The work on the bacterial systems was supported by PNNL's Biological Systems Initiative (BSI) LDRD Program. The development of the PASS methodology was supported by BSI and the Department of Energy Office of Biological and Environmental Research Program under Grant 22342 KP-14-02-01. The research was performed in the Environmental Molecular Sciences Laboratory (a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research) located at Pacific Northwest National Laboratory, and operated by Battelle for DOE under contract DE-AC06-76RL01830. Jerry L. Phillips is acknowledged for preparing both the TSB growth medium and CN32 cells.

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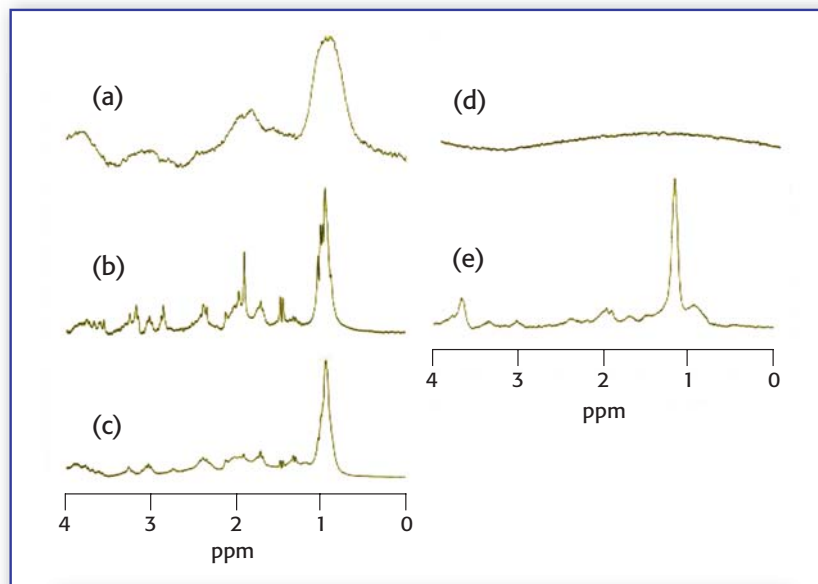


Figure 4. (a), (b): ^1H NMR spectra of *Shewanella putrefaciens* CN-32 bacteria attached to polystyrene beads (total volume $\sim 0.3\text{ cm}^3$), obtained in a static sample (a) and with 40 Hz PASS (b). (c): The 40-Hz ^1H PASS NMR spectrum of Tryptic Soy Broth surrounding polystyrene beads ($\sim 0.3\text{ cm}^3$). (d), (e): The ^1H NMR spectra of *Shewanella putrefaciens* CN-32 bacteria attached to quartz sand beads (total volume $\sim 0.3\text{ cm}^3$), obtained in a static sample (d) and with 100-Hz PASS (e). The PASS spectra shown in (b) and (c) were acquired using sixteen evolution steps with 32 (b), and 96 (c) scans and a recycle delay time of 1 s. The spectrum shown in (e) was acquired using 32 evolution steps, 64 scans and a recycle delay time of 2 s. All the PASS spectra are center band spectra.

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