In vivo metabolic analysis of Pseudomonas aeruginosa live bacteria using High Resolution Magic Angle Spinning NMR spectroscopy

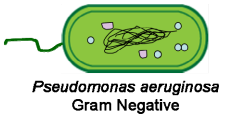
V. Righi[1,2], C. Constantinou[3], M. Kesarwani[3], L. G. Rahme[3], and A. A. Tzika[1,2]

[1]NMR Surgical Laboratory, Department of Surgery, Massachusetts General Hospital and Shriners Burns Institute, Harvard Medical School, Boston, MA, United States, [2]Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Athinoula A. Martinos Center for Biomedical Imaging, Boston, MA, United States, [3]Molecular Surgery Laboratory, Department of Surgery, Massachusetts General Hospital and Shriners Burns Institute, Harvard Medical School, Boston, MA, United States

Introduction– *Pseudomonas aeruginosa* (PA) is a human opportunistic pathogen responsible for chronic and acute infections, and is a major cause of morbidity and mortality in cystic fibrosis (CF) patients. Bacterial cell walls have a very complicated structure, consisting of integrated macromolecules such as carbohydrates, lipids, and proteins. This structure is highly heterogeneous among individual bacterial cells, due to constant biosynthesis, assembly, disassembly, and turnover. To understand the bacterical cell wall structures destructive methods have been used to analyze the individual components. These *in vitro* results may not faithfully reflect the native structural and conformational information. Recently, cell NMR spectroscopy has gained recent popularity (1). ^{1}H High Resolution Magic Angle Spinning (HRMAS) NMR can determine bacterial structure in detail. Here, ^{1}H HRMAS NMR was applied to *Pseudomonas aeruginosa* to determine the metabolites in living cells.

Materials and Methods– PA14 cells were grown in LB. 10ml of culture at OD 600_{nm} 2.0 was centrifuged and the pellet washed once with PBS. The bacterial samples were weighed and the ~60mg of cells were introduced into the 4mm zirconia rotor and 10 µl D_2O (deuterium lock reference) containing 50 mM TSP (trimethylsilyl propionic-2,2,3,3-d_4 acid, M_w=172, δ=0ppm, external chemical shift reference) was added to the rotor with the sample. ^{1}H HRMAS experiments were performed on a Bruker Bio-Spin Avance NMR spectrometer (600.13 MHz) using a 4mm triple resonance (^{1}H, ^{13}C, ^{2}H) HRMAS probe (Bruker). The temperature was controlled at 4°C by a BTO-2000 unit in combination with a MAS pneumatic unit (Bruker). Samples were spun at 3k Hz and one dimensional (1D) proton spectra were acquired using a water-suppressed spin-echo Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence [90˚-(τ-180˚-τ)n]. The CPMGpr was performed using 2.5 s water presaturation during relaxation delay, 1 ms echo time (τ) and 100 ms total spin-spin relaxation delay (2nτ), 8 kHz spectral width, 32k data points, 64 scans. The detection of metabolites was obtained, not only through 1D ^{1}H CPMG spectra, but also through selected 2D experiments ^{1}H,^{1}H TOBSY (TOtal Through-Bond SpectroscopY) (2) and their assignment was confirmed by comparison with published data (3). TOBSY acquisition parameters were: 2 k points direct dimension (13 ppm spectral width), 200 points indirect dimension (7.5 ppm spectral width), 8 scans with 2 dummy scans, 1 s water pre-saturation, 2 s total repetition time, 45 ms mixing time and total acquisition time 45 min. Concentrations of the metabolite were calculated using MestReC software (Mestrelab Research) an automated fitting routine based on the Levenberg-Marquardt algorithm was applied after manual peak selection, adjusting peak positions, intensities, linewidths and Lorentzian/Gaussian ratio until the residual spectrum was minimized.

Results– Fig. 1 shows the PA cells and their complex membrane, and Fig. 2 shows a representative 1D CPMG HRMAS NMR spectrum of live bacterial cells. Several metabolites can be detected using the CPMG sequence; it is fast and can provide important information. However, there is peak overlap and it is thus difficult to assign peaks to specific molecules. The 2D TOBSY HRMAS experiment was performed in order to complete the metabolic profiling of bacteria cells (figure 3). We identified from the analysis of 1D and 2D spectra 25 metabolites in *P. aeruginosa* strain PA14. A variety of informative metabolites were detected, (i.e., capsular polysaccharides structure, signals due to their N-Acetyl signal (2.02÷2.33/ 4.10÷4.33 ppm) (4); these along with the phospholipids signal can be very informative about the consistency of bacterial membrane. Other metabolites like betain compounds were detected. These molecules are well known to act as osmoprotectors and recently shown to preserve L-homoserine lactone (HSL) and 2-heptyl-4-quinolone (HHQ) bacterial cell-to-cell signaling molecules production (5).



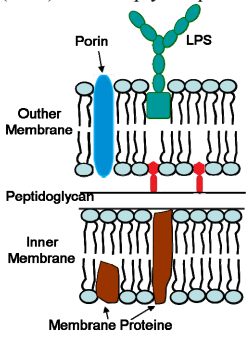


Figure 1. *Pseudomonas aeruginosa* and the bacterial membrane

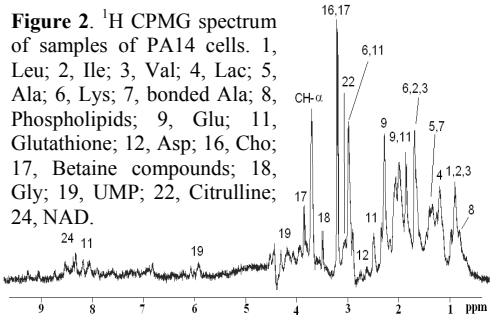


Figure 2. ^{1}H CPMG spectrum of samples of PA14 cells. 1, Leu; 2, Ile; 3, Val; 4, Lac; 5, Ala; 6, Lys; 7, bonded Ala; 8, Phospholipids; 9, Glu; 11, Glutathione; 12, Asp; 16, Cho; 17, Betaine compounds; 18, Gly; 19, UMP; 22, Citrulline; 24, NAD.

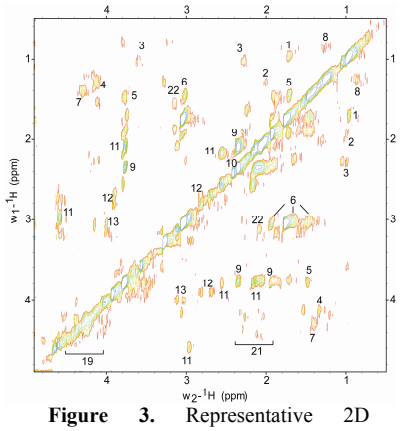


Figure 3. Representative 2D HRMAS NMR spectrum from PA14. As shown in TOBSY MR spectrum, the capsular polysaccharides, are detected due to their N-Acetyl signal [21], (2.02÷2.33/ 4.10÷4.33 ppm). Also, glutathione [11], a major cell antioxidant, is detected with higher resolution than in 1D.

Discussion– Use of both 1- and 2-dimensional ^{1}H HRMAS NMR results in a powerful technique in a variety of *in vivo* studies, including live bacterial cells in this study. Multidimensional HRMAS NMR using intact bacterial cells represents a promising method that could provide *in vivo* information of metabolomics in live bacteria. To this end, it can be complementary to existing chemical and biological methods. This technique may prove to be a helpful tool in gene function validation, the study of pathogenesis mechanisms, the classification of microbial strains into functional/clinical groups and the testing of anti-bacterial agents.

References

1. Li, W. Analyst. 131(7):777, 2006.
2. Andronesi, O, Mintzopoulos, D, Struppe, J, Black, PM, Tzika, AA. J Magn Reson. 193(2): 251, 2008.
3. Fan, TW-M. Prog. Nucl. Magn. Reson. Spec. 28:161, 1996.
4. Westman, EL, McNally, DJ, Charchoglyan, A, Brewer, D, Field, RA, Lam, JS. J Biol Chem. 284(18):11854, 2009.
5. Bazire, A, Diab, F, Taupin, L, Rodrigues, S, Jebbar, M, Dufour, A. Open Microbiol J. 3:128, 2009.