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Chemical fixation methods for Raman spectroscopy-based analysis of bacteria

Daniel S. Read [a,*], Andrew S. Whiteley [b]

[a] Centre for Ecology & Hydrology, Benson Lane, Crowmarsh Gifford, Wallingford OX10 8BB, UK
[b] School of Earth and Environment, The University of Western Australia, Stirling Highway, Crawley, Western Australia 6065, Australia

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ABSTRACT

Preservation of biological samples for downstream analysis is important for analytical methods that measure the biochemical composition of a sample. One such method, Raman microspectroscopy, is commonly used as a rapid phenotypic technique to measure biomolecular composition for the purposes of identification and discrimination of species and strains of bacteria, as well as investigating physiological responses to external stressors and the uptake of stable isotope-labelled substrates in single cells. This study examines the influence of a number of common chemical fixation and inactivation methods on the Raman spectrum of six species of bacteria. Modifications to the Raman-phenotype caused by fixation were compared to unfixed control samples using difference spectra and Principal Components Analysis (PCA). Additionally, the effect of fixation on the ability to accurately classify bacterial species using their Raman phenotype was determined. The results showed that common fixatives such as glutaraldehyde and ethanol cause significant changes to the Raman spectra of bacteria, whereas formaldehyde and sodium azide were better at preserving spectral features.

1. Introduction

Raman microspectroscopy is a method commonly used for the phenotypic measurement of biological samples, ranging from individual cells to complex structures such as biofilms and tissues (Huang et al., 2010a,b; Schuster et al., 2000). Measurement of the inelastic scattering of light (Raman scattering) can be used to non-destructively determine the molecular composition of a biological sample (Schie and Huser, 2013). The Raman spectrum can provide a spectroscopic fingerprint that can measure the molecular composition of cells, comprising major biological molecules including proteins, amino acids, lipids, polysaccharides, nucleic acids and nucleobases (Huang et al., 2010a).

One of the most frequent applications of Raman spectroscopy in microbiology is to measure the cellular composition (the phenotype) for the purposes of species/strain identification. This approach has previously been used to identify and discriminate between species and strains of fungi (De Gussem et al., 2007), algae (Huang et al., 2010a,b), viruses (Driskell et al., 2010) and most frequently bacteria (Palchaudhuri et al., 2011; Read et al., 2013). As well as a rapid identification tool, Raman has been used to examine the phenotypic and physiological changes that occur with exposure to stressors in the form of pollutants such as ionic metals (Walter et al., 2012), metal nanoparticles (Cherchi et al., 2011), organic pollutants (Daniel et al., 2008; Singer et al., 2005), antibiotics (Escoriza et al., 2007), and pharmaceuticals (Wharfe et al., 2010). Raman has also been used to measure the concentration and spatial distribution of cellular metabolites such as algal lipids (Wu et al., 2011) and pigments such as carotenoids (Tao et al., 2011) and chlorophyll (Huang et al., 2010b). Finally, there is an emerging application applying Raman microspectroscopy as a tool for stable isotope probing (SIP) to monitor substrate utilisation by single bacterial cells (Huang et al., 2004, 2007).

As with all analytical techniques that measure phenotypic characteristics (such as proteomics, metabolomics and lipidomics), methods for sample handling and preservation of samples for later analysis are of critical importance. As Raman spectroscopy measures the molecular composition of the cell, it is important to use preservation methods that cause minimal changes to the composition and arrangement of molecules that make up the Raman fingerprint. Unless cells are suitably fixed, autolysis by intracellular enzymes can denature proteins and dephosphorylate mononucleotides, phospholipids and proteins (Gazi et al., 2005), potentially altering the Raman fingerprint.

Previous work examining the role of sample handling and preservation techniques on the Raman spectra of eukaryotic tissues has highlighted method-dependant spectral alterations. These include the effects of ethanol and glycerol on bone samples (Yeni et al., 2006), snap freezing in liquid nitrogen on porcine prostate tissue (Candefjord et al., 2009), formaldehyde or methanol fixation in leukaemia cells lines (Chan et al., 2009), formaldehyde, desiccation and air drying on human cell lines (Mariani et al., 2009), desiccation on human embryonic stem cells (Konorov et al., 2011) and formalin or Carnoy's fixative on human cell lines (Meade et al., 2010). There have been studies examining fixation

* Corresponding author.
E-mail address: dasr@ceh.ac.uk (D.S. Read).

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and inactivation induced effects on bacterial spectra, but these have focussed specifically on purple non-sulfur bacteria (Kniggendorf et al., 2011) and endospore forming species (Stockel et al., 2010).

The objective of the current study was to investigate the influence of a number of common chemical fixatives on the Raman spectra of species of bacteria representing a range of different Raman phenotypes. Here we have examined the effect that each fixative has on the Raman spectra of six species of bacteria, followed by an examination of the influence of each fixative on the ability to correctly identify each bacterial species based on their Raman spectra.

2. Materials and methods

2.1. Bacterial isolates and culturing

Six bacterial species, selected to represent a range of differing phenotypes, were purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany). These were; *Escherichia coli* (ATCC 1775), *Bacillus subtilis* subsp. *subtilis* (ATCC 6051), *Pseudomonas fluorescens* (ATCC 13525), *Pseudomonas aeruginosa* (ATCC 10145), *Micrococcus luteus* (ATCC 4698), and *Janthinobacterium lividum* (ATCC 12473). All strains were checked for purity by streaking onto LB agar (Sigma Aldrich, UK) and cultured overnight at 28 °C. Single colonies were picked and subcultured in 5 ml of LB broth (Sigma, UK) with shaking at 180 rpm. Each culture was diluted to an OD_{600} of 0.5 and used to inoculate 180 ml of LB broth for each treatment and again grown overnight (16 h) at 28 °C with shaking at 180 rpm. The cell suspension was well mixed, and divided into six aliquots of 30 ml, one for each of the fixation methods and then further divided into three aliquots of 10 ml to provide technical fixation replicates. To remove the influence of the culture media on the fixation methods, each cell suspension was centrifuged for 5 min at 5000 *g*, the supernatant was removed using a pipette and cells were re-suspended in ice cold ×1 PBS.

2.2. Fixation and sample handling

Five methods of chemical cell fixation were compared, including fixation in 70/30 (vol:vol) mix of ethanol (EtOH) and molecular grade water, a solution of 2.0% neutral buffered formaldehyde (CH_2O) made fresh from paraformaldehyde (adjusted to pH 7.2), a solution of 1.0% glutaraldehyde ($CH_2(CH_2CHO)_2$), a solution of 1.0% formaldehyde and 0.05% glutaraldehyde, and finally a solution of 10% (w/v) sodium azide (NaN_3). All chemicals were purchased from Sigma-Aldrich, UK. Cell pellets were re-suspended in each fixative and allowed to fix for 1 h at room temperature before being washed, pelleted and re-suspended in ice cold MQ H_2O three times as before. The supernatant was removed a final time using a pipette to leave a cell pellet. The control sample consisted of unfixed cells frozen immediately after washing. The samples were then prepared for analysis by Raman spectroscopy by spotting 10 μl of the cell pellet from each replicate and treatment onto spectroscopy grade CaF_2 slide (Crystran, UK) and dried in a laboratory desiccator at room temperature for 30 min.

2.3. Raman microspectroscopy

Raman spectroscopy was conducted on a Horiba LabRAM HR800 Raman microspectrometer (Horiba Scientific, UK) equipped with an Olympus BX-41 microscope and an Andor electronically cooled CCD detector. The dried cell mass was visually focused on using a 100×/0.9 numerical-aperture Olympus M Plan air objective and a CCD camera, viewed on LabSpec v5. The samples were illuminated with a 532-nm Nd:YAG laser and the incident laser power was adjusted to 5–8 mW. The signal was optimized by adjusting the laser focus using the real-time readout of the Raman signal, before acquiring the spectrum between 211 cm^{-1} and 1894 cm^{-1}, with 1022 data points (~1.5 cm^{-1} per point). Each spectrum consisted of two averaged 30 s exposures. Cosmic spikes were automatically removed using LabSpec v5 software (Horiba Scientific, UK). Raman spectra were collected from 4–8 spatially offset points within each dried bacterial spot for each replicate, to give a total of 12–24 spectra per treatment.

2.4. Data analysis

Raw spectra were concatenated to between 400 cm^{-1} and 1800 cm^{-1} wavenumbers, and the data normalized (area under spectra to 100 units) using LabSpec V5. The data analysis had two main objectives; firstly to examine the relative influence of fixation on the Raman spectra of the different bacterial species. Difference spectra were generated by subtracting the average spectra of each treatment from the average control spectra. Differences in the structure and shape of the treatment vs. the control are highlighted in deviations from the zero line. To further explore fixation-induced changes in spectral composition, Principle Components Analysis (PCA) was used to examine the relationships of all the treatments for each spectrum. PCA was conducted in the R programming environment (R Core Team, 2013) using the package "ChemometricsWithR" (Wehrens, 2012). The second objective was to examine the influence of fixation on the ability to accurately discriminate between bacterial species using their Raman spectra. Hierarchical Cluster Analysis (HCA) in R was used to create a dendrogram for each treatment, showing unsupervised clustering of the spectra replicates for each strain. Additionally, the accuracy of species discrimination was assessed using Linear Discriminant Analysis (LDA) in the R package "MASS" (Venables and Ripley, 2002) and the apparent error rate visualized and assessed using the KlaR package (Weihs et al., 2005).

3. Results and discussion

Due to very high levels of autofluorescence, it was not possible to collect a spectrum from *J. lividu*m fixed with a formaldehyde and glutaraldehyde solution, so these data were excluded from further analysis. Fig. 1 shows representative Raman spectra from unfixed samples of each of the six species. Whilst the overall structure and composition of the spectra are broadly similar, there are some differences caused by variation in the fluorescence background for each species. Although there was a concordance in terms of the presence/absence of specific

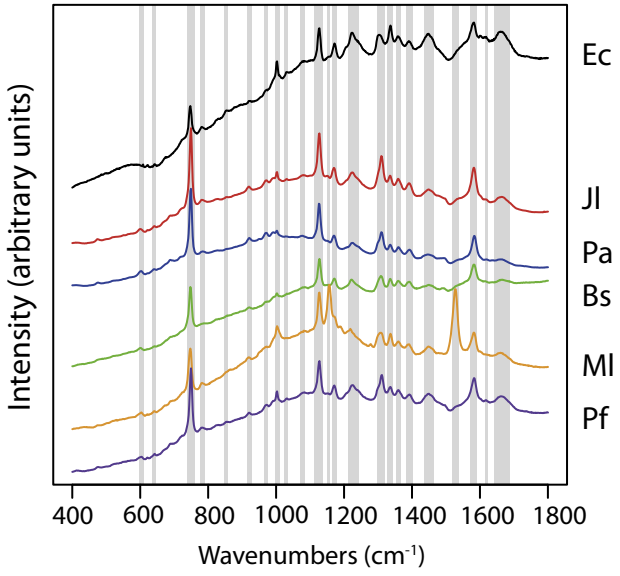

Fig. 1. Representative Raman spectra for each species of bacteria used in this study; *Escherichia coli* (Ec), *Janthinobacterium lividum* (Jl), *Pseudomonas aeruginosa* (Pa), *Bacillus subtilis* (Bs), *Micrococcus luteus* (Ml) and *Pseudomonas fluorescens* (Pf). Major peaks are highlighted with grey bars.