

Technical Note

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Label-Free Discrimination of Rhizobial Bacteroids and Mutants by Single-Cell Raman Microspectroscopy

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ABSTRACT

Symbiotic rhizobia in legume account for a large portion of nitrogen fixation in the biosphere. Nitrogen fixation is an energy-demanding process requiring tight control of metabolism and redox state. It is of great interest to understand the bacteroid differentiation process and the roles of energy storage molecules, such as glycogen and polyhydroxybutyrate (PHB), in maintaining the *Rhizobium*-legume symbioses. Traditional biochemical assays for checking phenotypic changes of mutants require a large volume of starting materials, which is difficult for unculturable, terminally differentiated bacteroids. Here we present a label-free technique that allows the identification and characterisation of phenotypic changes of bacteria at the single-cell level. This work demonstrates the application of single-cell Raman spectra (SCRS) to differentiate *Rhizobium leguminosarum* bv. *viciae* wild-type and mutants under different conditions. We found symbiotically differentiated bacteroids and free-living bacteria differed primarily at a Raman biomarker, cytochrome c, corresponding to a bacteroid-specific terminal oxidase. We demonstrated that, for the first time, SCRS were able to link phenotypic changes and specific genetic mutants, in this case, single and double mutations in synthesis of carbon storage molecules glycogen and polyhydroxybutyrate (PHB). By analysing SCRS of these mutants, it provides insights into metabolite production and carbon regulatory network of rhizobia.

Key words: Single cell, Raman, microscope, phenotype, mutants, *Rhizobium*, nitrogen fixation, cytochrome c, glycogen, polyhydroxybutyrate, PHB.

INTRODUCTION

Nitrogen fixation by *Rhizobium*-legume symbioses provides a significant fraction of the available nitrogen in the biosphere, making it agronomically and ecologically important¹. Rhizobia are hosted by a plant in an optimal low-oxygen environment required for nitrogen fixation which is the root nodule². Inside the nodule, bacteria differentiate into bacteroids, shutting down motility and reproduction and upregulating expression of nitrogen-fixation machinery and other proteins to maintain nitrogen fixation³. *Rhizobium leguminosarum* oxidises a wide range of carbon sources and stores excess carbon and reductant in the forms of polyhydroxybutyrate (PHB) and glycogen. These storage polymers can be detected in rhizobia, including in their differentiated bacteroid state^{4, 5}. In *R. leguminosarum* bv. *viciae*, PHB is found inside the infection threads carrying undifferentiated rhizobia into their host plant cells⁶. This PHB is thought to be degraded to fuel bacteroid differentiation process^{4, 6}. In developed bacteroids within pea nodules, glycogen is thought to be the main carbon storage compound used to relieve redox stress under conditions where carbon is in excess⁴.

To examine these biologically significant molecules in the process of bacteroid development as well as in symbiotic efficiency, traditional bulk chemical analysis can be used to measure the bulk abundance level of the molecules. In the case of rhizobia, bacteroids are terminally differentiated and cannot be cultured outside of the nodule. This requests large volumes of plant material to carry out biochemical assays. Furthermore, a bulk measurement approach could overlook important information on cellular heterogeneity which results from stochastic gene expression and various physiological states, such as between infection-thread bacteria and differentiated N₂-reducing bacteroids. Therefore, evaluation and quantification at the intracellular level has been difficult and dependent on molecular tools such as fluorescence *in situ* hybridization (FISH) or quantitative-polymerase chain reaction (q-PCR)⁷. These genetic-based techniques, however, can only be applied to *in vitro* and pre-known genetic conditions, and often require external labelling.

Here we applied single-cell Raman spectra (SCRS) to reveal bacterial phenotypic changes at the single-cell level. SCRS provide label-free and intrinsic information by measuring molecular vibrational fingerprints of single cells⁸. In this study, SCRS are used to discriminate physiological states of *R. leguminosarum*, and for the first time, differentiate genetically defined mutants from their wild-types. This study demonstrated that SCRS are able to detect relative abundancy of intracellular biomolecules in single cells, such as cytochrome c, glycogen and PHB. Notably, with the help of comprehensive biochemical

fingerprints, analysis of SCRS can lead to unexpected new discovery of phenotypic changes in cells.

EXPERIMENTAL SECTION

Strains and culture conditions. Strains used in this study are detailed in Table S-1. Free-living *R. leguminosarum* bv. *viciae* strains were grown at 28°C in TY media⁹. The wild-type strains Rlv3841 and RlvA34 were grown in free-living laboratory culture and isolated as bacteroids from *Pisum sativum* nodules. Glycogen synthase (*glgA*) and PHB synthase (*phaC*) mutants generated according to Lodwig et al.⁴ and Terpollili et al.⁵ were inoculated on *P. sativum* and bacteroids harvested. Procedure of bacteroid preparation and information of the mutants can be found in Supporting Information.

Single-cell Raman spectra (SCRS) measurements. Cells were spread onto an aluminium-coated slide and observed under a 100×/0.9 microscope objective. Single-cell spectra were acquired using an HR Evolution confocal Raman microscope (Horiba) equipped with a 532 nm neodymium-yttrium aluminium garnet laser and 600 grooves/mm diffraction grating. Acquisition time was 20 s per spectrum with 0.5 mW laser power and 1 μm^2 laser spot. For each sample, 30-50 single cells were analysed.

Data Processing and Multivariate Analysis of Raman spectra. All spectra were pre-processed by cosmic ray correction and polynomial baseline fitting using LabSpec 5. Spectral normalisation was done by vector normalisation of the entire spectral region. Principal component analysis (PCA) was performed using MultiVariate Statistical. Quantification of intracellular biomolecule concentration was done by integrating individual Raman band. Box plots showing quantification distribution were plotted using Microsoft Excel and Real Statistics Data Analysis Tool. The rectangle in the box plots represents the second and third quartiles with a line inside representing the median. The lower and upper quartiles are drawn as lines outside the box.

RESULTS AND DISCUSSION

Single-cell Raman spectra (SCRS) distinguished cells in free-living and bacteroid states.

Fig.1A shows the SCRS of *Rhizobium leguminosarum* bv. *viciae* wild-type Rlv3841, as symbiotic bacteroids in nodules and as free-living bacteria in culture. Raman characteristic bands of cytochrome c at 749 (pyrrole breathing mode), 1128 ($\nu(\text{CN})$ stretching vibrations), 1312 ($\delta(\text{CH})$ deformations), and 1584 cm^{-1} ($\nu(\text{CC})$ skeletal stretches)^{10,11,12} were observed in

bacteroid cells isolated from nodules, whilst these bands were significantly decreased in the free-living cells incubated in rich media. Cytochrome c is a hemeprotein absorbing visible light at a wavelength of around 530 nm, thus using a 532 nm excitation laser light generates a resonance Raman spectrum of the heme prosthetic group¹². This significantly enhances the Raman scattering sensitivity compared to detection of other non-resonant intracellular constituents. These cytochrome subunits correspond to high-affinity cytochrome cbb₃ oxidase FixNOQP, which is used to maintain respiration within the microaerobic environment required for nitrogenase activity¹³. The SCRS result is in a good agreement with physiology observation of *R. leguminosarum* bv. *viciae*, as the cytochrome c is expressed in *R. leguminosarum* bv. *viciae* under the low-oxygen conditions within the nodule, and it should be weak or absent in the free-living, aerobic culture¹⁴.

Spectral quantification of four characteristic Raman bands of cytochrome c in both strains has been enabled by integrating individual Raman bands. This allows semi-quantification of concentrations of intracellular cytochrome c within bacteroids and bacteria. It shows that cytochrome c bands were significantly higher in cells isolated from nodules than those grown in culture ($p < 0.001$) (Fig. 1B). It is notable that the relative intensity at 780 cm⁻¹ in SCRS of rhizobial bacteroids (Fig. 1A) was significantly decreased compared to those in free-living culture ($p < 0.001$) (Fig. S-1). Biological assignment of the band at 780 cm⁻¹ is cytosine and uracil ring breathing¹⁵, which implies that nucleic acid level in free-living bacteria is higher than those in bacteroid states. Further study should be done to investigate the differences at the nucleic acid level. Subsequent PCA analysis shows a classification of two physiological states, indicating a clear difference between their phenotypic profiles (Fig. S-2A). In PCA loading plots (Fig. S-2B and S-2C), Raman wavenumbers responsible for the most significant loadings at axis 1 and axis 2 coincide with the cytochrome c wavenumbers, indicating that cytochrome c is the main factor contributing to this classification. These results suggest that the SCRS are able to reflect phenotypic profiles of *R. leguminosarum* in free-living and bacteroid states. It provides an insight of bacterial cells in different physiological states without external labelling or pre-known knowledge of specific biomolecule expression.

Single-cell Raman spectra (SCRS) link phenotypic changes of cells and gene knockouts.

SCRS were obtained for wild-type strain Rlv3841 and its derived mutant LMB816 (*phaC1::Tn5/phaC2::Ω*), and wild-type strain RlvA34 and its derived mutants RU1448 (*glgA::TnB60*) and RU1478 (*glgA::TnB60/phaC::Ω*) (see Table S1 for details). Strain RlvA34 has a single gene encoding PHB synthase, *phaC*⁴, whilst Rlv3841 has two PHB

synthases, encoded by *phaC1* (encoded on the chromosome) and a type III PHB synthase encoded by *phaC2-phaE* (encoded on the 'sym' plasmid, and expressed only in bacteroids)⁵; all PHB mutants in the Rlv3841 background had both *phaC* genes mutated. The ability to synthesise glycogen or/and PHB is expected to be compromised in these mutants (*glgA* - glycogen synthase mutation, *phaC/phaC1phaC2* - PHB synthase mutation).

SCRS reveal clear phenotypic changes in mutants due to loss of the ability to synthesise carbon storage molecules. Fig. 2 shows the Raman spectra of the wild-type Rlv3841 with one derived mutant and the wild-type RlvA34 with two derived mutants. Two highlighted bands at 1050 and 1735 cm⁻¹ can be assigned to $\nu(\text{C=O})$ stretching vibrations of glycogen¹⁶ and $\delta(\text{C=O})$ stretching vibrations of ester groups in PHB, respectively¹⁵. By measuring pure glycogen, another characteristic band associated with pure glycogen was also identified at 850 cm⁻¹ (Fig. S-3). Raman bands at 850 and 1050 cm⁻¹ in SCRS are chosen as glycogen biomarkers because they have little overlap with other bacterial bands that typically exist in the fingerprint region. Comparing SCRS of RlvA34 with RU1448 that contains defective *glgA* (glycogen synthase), a clear disappearance at both 850 and 1050 cm⁻¹ (glycogen band) in RU1448 (*glgA*) indicates that glycogen synthesis was compromised (Fig. 2). Compared to their corresponding wild-types, a decrease in the characteristic PHB Raman band at 1735 cm⁻¹ indicates that PHB synthesis in the mutants LMB816 (*phaC1/phaC2*) and RU1478 (*glgA/phaC*) have been disrupted, which corresponds to the different mutations of PHB synthases. A reduction in 850 and 1050 cm⁻¹ (glycogen) and a disappearance of 1735 cm⁻¹ (PHB) in the Raman spectra of RU1478 (*glgA/phaC*) are in a good agreement that this strain is mutated in both glycogen and PHB synthases.

Statistical analysis validates the link between gene knockouts and phenotypic changes observed by SCRS.

Statistical analysis of SCRS from 30-50 randomly chosen single cells has been applied to quantify the relative abundance of the intracellular polymers *in situ* and confirm the link between gene knockouts and phenotypic changes in mutants. Fig. 3 shows the abundance distributions of different levels of intracellular carbon storage polymers by individually examining Raman bands at 1735 cm⁻¹ (PHB) and 1050 cm⁻¹ (glycogen). 850 cm⁻¹ band has also been evaluated in Fig. S-4, exhibiting similar statistical patterns as the band at 1050 cm⁻¹.

Fig. 3A shows a statistical distribution of intercellular PHB levels in bacteroid single cells. It reveals a remarkably lower PHB accumulation ($p < 0.001$) in mutants LMB816 (*phaC1/phaC2*) and RU1478 (*glgA/phaC*), all of which completely prevent PHB synthase (either *phaC* in the

A34 background, or *phaC1/phaC2* in the Rlv3841 background). A correlation between PHB and glycogen, which both serve as carbon storage compounds, is also found in both SCRS (Fig. 2) and single-cell statistics (Fig. 3A). A single gene mutation of glycogen synthesis in the mutant RU1448 (*glgA*) leads to a decrease in PHB level ($p < 0.001$). This suggests a link between biosynthesis of these two carbon storage molecules; in the absence of glycogen stores, PHB was more rapidly broken down in order to provide carbon or reductants.

In Fig. 3B and S-4, the glycogen Raman biomarkers at 850 and 1050 cm^{-1} were examined individually to study the cellular glycogen level and similar results on glycogen quantification proved the integrity of these two biomarkers responsible for glycogen identification. It is observed that RU1448 (*glgA*) and RU1478 (*glgA/phaC*) have a significant decrease in the glycogen level, compared to their wild-type strain RlvA34 in bacteroid state ($p < 0.001$). Both single (RU1448) and double (RU1478) mutants lose their ability to store glycogen in the carbon-excessive bacteroid state, which is in good agreement with a previous report⁴. Interestingly, SCRS (Fig. 2) and statistical quantification (Fig. 3B) of the PHB mutant LMB816 (*phaC1/phaC2*) display a glycogen level lower than the wild-type Rlv3841 ($p < 0.001$), supporting the hypothesis that biosynthesis of these storage molecules is linked. This is different from *Rhizobium tropici* and *Rhizobium etli*, where mutants in PHB synthesis had increased glycogen accumulation¹⁷. Our results show linkage between these two storage molecules with a mutant in glycogen synthesis also having reduced PHB (Fig. 3A), and mutants in PHB synthesis having reduced glycogen accumulation (Fig. 3B). The production of carbon storage molecules may be tightly coupled in *R. leguminosarum* bv. *viciae*, under the control of a so-far unknown global carbon regulatory network acting to maintain redox balance.

Single-cell analysis reveals metabolic profiles of uncultured R. leguminosarum

SCRS not only reveal phenotypic differences among *R. leguminosarum* bv. *viciae* in different physiological states and its mutants using characteristic Raman biomarkers, but also shows a distribution of phenotypic heterogeneity in individual bacterial cells (Fig. 1B and 3). Individual biochemical assays require a large volume of starting material, which can be difficult in the case of terminally differentiated bacteria that have lost their ability to reproduce, such as bacteroids. Bacteroids within a single nodule have extremely heterogeneous populations in which important information can be averaged out in bulk chemical analysis. Single-cell assays could also be beneficial in the case of organisms that are difficult to culture, such as unknown soil bacteria or marine microorganisms. Many

biochemical assays only focus on one specific molecule analysis, and so collective protocols would be required to create a basic metabolic profile. Other technologies such as fluorescent labelling require information of DNA/RNA or protein in order to design fluorescent tag. SCRS analysis allows semi-quantitative analysis of several macromolecules within a single experiment without prior knowledge. This can provide novel insights into productions of different metabolites and has the potential of making new scientific discoveries of cell phenotypes. More accurate quantification can be achieved if used in conjunction with other analytical techniques such as gas chromatography-mass spectrometry, providing with a standardised reference.

CONCLUSIONS

SCRS of the symbiotic diazotroph *R. leguminosarum* by *viciae* in free-living culture and terminally differentiated bacteroids in pea nodules primarily differ, as free-living cells lack cytochrome c Raman bands present in the cbb₃ complex. Raman band at 780 cm⁻¹ also indicates a difference at nucleic acid level. SCRS and PCA analysis reflect their phenotypic changes due to different physiological states despite being genetically identical. This study also demonstrates that for the first time Raman measurements are able to discriminate mutants of *R. leguminosarum* by *viciae*, including both single and double mutants. Genetic mutants of important carbon storage molecules (e.g. PHB and glycogen) can be confirmed and semi-quantified by analysing characteristic Raman biomarkers (e.g. PHB and glycogen). These results confirm that SCRS are label-free and non-destructive biochemical profiles of single cells, able to detect phenotypic differences and produce intrinsic fingerprints. Furthermore, to where genomics cannot reach, SCRS analysis can be potentially applied to a wide range of isogenic cells or mutants with heterogeneous behaviours.

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SUPPORTING INFORMATION

Details on additional experimental, spectra and statistics are included in Supporting Information.

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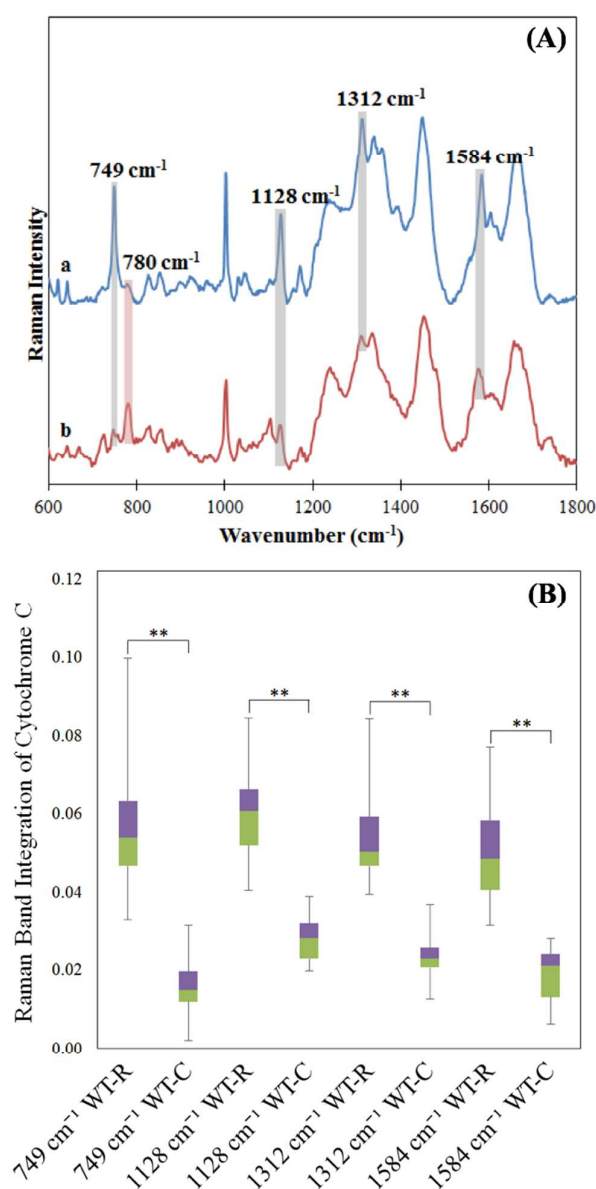


Figure 1. (A) Averaged single-cell Raman spectra (SCRS) of the wild-type Rlv3841 as (a) symbiotic bacteroids in root nodules and as (b) free-living bacteria in culture, differ primarily at 749, 1128, 1312 and 1584 cm⁻¹, which can be assigned to cytochrome c. (B) Box plot of single-cell band integration shows statistical distribution of concentrations of intracellular cytochrome c, in which WT-R represents Rlv3841 in root nodule and WT-C represents Rlv3841 in culture. ** indicates statistical significance ($p < 0.001$).

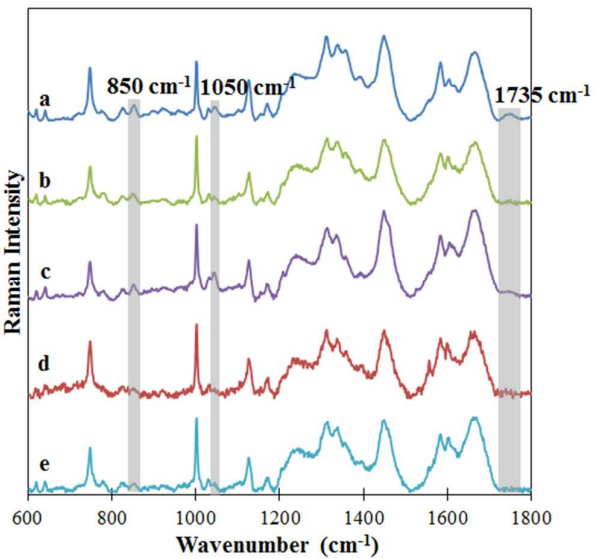


Figure 2. Averaged SCRS of *R. leguminosarum* in bacteroid state: (a) wild-type Rlv3841 and (b) its derived mutant LMB816 (*phaC1/phaC2*) and (c) wild-type RlvA34 and its derived mutants (d) RU1448 (*glgA*) and (e) RU1478 (*glgA/phaC*) with highlighted bands at 850 cm⁻¹ (glycogen), 1050 cm⁻¹ (glycogen) and 1735 cm⁻¹ (PHB).

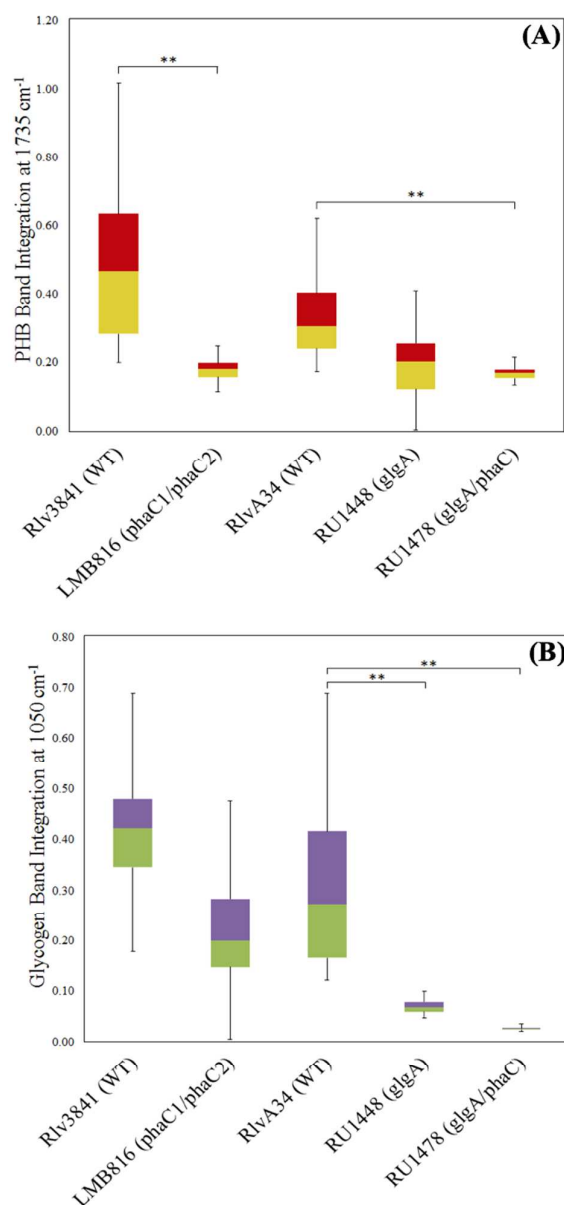
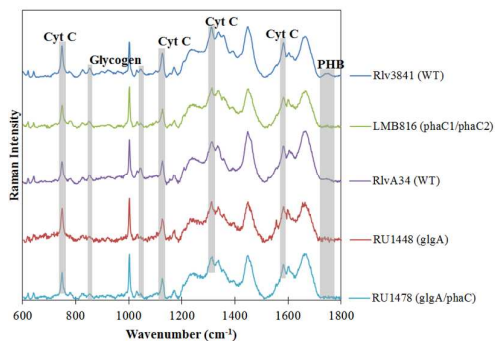


Figure 3. Box plots of statistical distribution of concentrations of intracellular A) PHB at 1735 cm⁻¹ and B) glycogen at 1050 cm⁻¹, calculated by integration of individual Raman band. ** indicates statistical significance (p<0.001) between wild-type Rlv3841 or RlvA34 and their derived mutants.



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