Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function

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Summary

We have coupled fluorescence in situ hybridization (FISH) with Raman microscopy for simultaneous cultivation-independent identification and determination of $^{13}$C incorporation into microbial cells. Highly resolved Raman confocal spectra were generated for individual cells which were grown in minimal medium where the ratio of $^{13}$C to $^{12}$C content of the sole carbon source was incrementally varied. Cells which were $^{13}$C-labelled through anabolic incorporation of the isotope exhibited red-shifted spectral peaks, the calculated ‘red shift ratio’ (RSR) being highly correlated with the $^{13}$C-content of the cells. Subsequently, Raman instrumentation and FISH protocols were optimized to allow combined epifluorescence and Raman imaging of Fluos, Cy3 and Cy5-labelled microbial populations at the single cell level. Cellular $^{13}$C-content determinations exhibited good congruence between fresh cells and FISH hybridized cells indicating that spectral peaks, including phenylalanine resonance, which were used to determine $^{13}$C-labelling, were preserved during fixation and hybridization. In order to demonstrate the suitability of this technology for structure–function analyses in complex microbial communities, Raman-FISH was deployed to show the importance of Pseudomonas populations during naphthalene degradation in groundwater microcosms. Raman-FISH extends and complements current technologies such as FISH-microautoradiography and stable isotope probing in that it can be applied at the resolution of single cells in complex communities, is quantitative if suitable calibrations are performed, can be used with stable isotopes and has analysis times of typically 1 min per cell.

Introduction

Microbial ecology has been revolutionized in the last two decades by the use of molecular methods, the technologies revealing an astonishing diversity of microorganisms on our planet (Hugenholtz et al., 1998; Rappé and Giovannoni, 2003; Gans et al., 2005; Sogin et al., 2006). However, in the vast majority of cases our insight is limited to environmentally retrieved 16S rRNA gene sequences and the deduced phylogenetic affiliation of the respective organisms. Consequently, a key challenge remains in deciphering the functional relevance of these organisms in situ, because most are representative of taxa which have yet to be cultured in the laboratory.

In order to address this shortfall, several technologies can be employed at a range of resolution, from the individual cell right through to the whole community. For single cells, developments based around fluorescence in situ hybridization (FISH) such as FISH-microautoradiography (FISH-MAR; Lee et al., 1999; Ouverney and Fuhrman, 1999; Gray et al., 2000), FISH combined with beta-microimaging (e.g. Gieseke et al., 2005) and FISH-secondary-ion mass spectrometry (FISH-SIMS; Orphan et al., 2001) provide high levels of spatial resolution for studying identity and ecophysiology in complex microbial assemblages. At the whole community level, technologies such as DNA and/or RNA stable isotope probing (SIP; Radajewski et al., 2000; Manefield et al., 2002a), the isotope array (Adamczyk et al., 2003) and SIP combined with a paramagnetic bead-capture protocol (MacGregor et al., 2006) provide a linkage between identity and function, but principally at the level of the genetic material (i.e. sequence recovery or detection).

Among these approaches, only the tools derived from FISH are based on microscopy and allow in situ visualization of complex structure–function relationships.
Single-cell resolution is highly desirable because it reveals links between the spatial architecture of microbial consortia and the ecophysiology of its members (Wagner et al., 2006 and references therein) and further provides visual in situ evidence to support function-specific community sequence retrieval strategies, such as that performed in DNA/RNA-SIP and isotope array studies (Adamczyk et al., 2003; Ginige et al., 2004; Whitley et al., 2006). However, technologies such as FISH-MAR currently employ radioactive tracers (e.g. $^{14}$C, $^3$H or $^{32}$P-labelled compounds) and can be labour intensive to quantify (Nielsen et al., 2003). Furthermore, radioactive-based technologies are not directly compatible with the majority of community sequence recovery strategies such as SIP, which use stable isotope tracers.

In theory, stable isotope enhancements to FISH-MAR and, thus, direct compatibility between these single cell techniques and community level SIP applications could be afforded by the use of multi-isotope imaging mass spectrometry (MIMS or nanoSIMS; Lechene et al., 2006; Kuyper and Jørgensen, 2007). Previous attempts at this have colocalized $^{13}$C stable isotope signatures with FISH probes in marine systems (Orphan et al., 2001; 2002) and attempts have been made to measure $^{13}$C incorporation for unhybridized cells in soil ecosystems (DeRito et al., 2005), both using SIMS analyses. However, recent investigations have indicated that Raman microspectroscopy might be an attractive alternative for in situ analyses of isotope-labelled microbial cells, due to reduced equipment costs and micron scale resolution.

Specifically, Raman microspectroscopic analyses allow the determination of chemical bonding patterns associated with biological molecules within individual microbial cells. The technology has recently been applied to study the biochemical composition of cells in bacterial pure cultures (Schuster et al., 2000a,b; Escoriza et al., 2006) and to identify bacterial pure cultures based on their cellular constituents (Huang et al., 2004; Jarvis et al., 2006). Of more significance for environmental microbiologists is that the incorporation of $^{13}$C stable isotope tracers into microbial cells causes significant changes in the observed resonance spectra, when compared with parent $^{12}$C-based spectra. These changes are principally due to the modification of bond vibrational states through the increased molecular mass contributed by the heavier isotopes, the vibrational frequency being inversely proportional to the square root of the atomic mass. This modification of bond vibrations due to interactions between heavy and light carbon isotopes has been termed a ‘red shift’ (Huang et al., 2004) due to the production of longer wavelengths within Raman spectra associated with $^{13}$C-labelled cells and constituents. Initial investigations also suggested that these spectral shifts correlate with the tracer content of the cells, and hence, Raman microspectroscopy seems to offer an inherently quantitative approach.

To this end, we report on the coupling of FISH to Raman microspectroscopy (‘Raman-FISH’), to facilitate stable isotope-based analyses of uncultured microorganisms at the single-cell level. This approach maintains the resolution of FISH-MAR, overcomes many of its limitations and provides an analysis bridge between highly resolved single-cell studies and population-level sequence retrieval strategies that use stable isotopes. We describe the development, calibration and application of Raman-FISH, initially using pure cultures and subsequently illustrate its potential with a proof-of-principle experiment in naphthalene degrading groundwater samples.

Results

$^{13}$C labelling of single microbial cells can be determined by characteristic spectral shifts

Confocal Raman microspectroscopy (with a resolution down to 1 μm) was found to be applicable at the single cell level and produced accurate and highly reproducible Raman vibrational spectra of microorganisms (Fig. 1). Key peaks which could be determined within the spectra include resonance regions associated with major cellular constituents such as proteins, lipids, nucleic acids and carbohydrates. In order to investigate the effect on
Raman spectra of $^{13}$C-incorporation during bacterial anabolism, we cultured *Pseudomonas fluorescens* on minimal medium with $^{12}$C6 or $^{13}$C6-glucose as the sole source of carbon. Comparing these subsequent spectra revealed ‘red-shifts’ in characteristic regions of the spectra (Fig. 1). Two affected vibrational regions were characteristic of protein and nucleic acid resonances while the strong resonance peak at 1003 cm$^{-1}$, which was observed to be red shifted by approximately 36 wave numbers down to 967 cm$^{-1}$, was characteristic of the essential amino acid phenylalanine. Furthermore, we observed that compound classes (e.g. proteins and nucleic acids) which are represented in multiple regions of the spectra displayed a greater shift at higher wave numbers (e.g. nucleic acid resonance approximately 1600 cm$^{-1}$ wave numbers vs. that at 800 cm$^{-1}$).

**Combining FISH with Raman microspectroscopy instrumentation and evaluating FISH probe fluorophores for use with Raman-FISH**

Raman microspectroscopy is traditionally a ‘bright field’ microscopic technology (Fig. 2A), which requires Raman analyses to be undertaken on specialist quartz slides in the absence of mounting media and coverslips. For this study a commercially available Raman microspectroscopy ‘bright field’ microscope chassis was upgraded to fluorescence imaging capabilities by the addition of epifluorescence light source, epifluorescence filter blocks and an extra fluorescence imaging camera. Subsequently, we performed parallel FISH experiments in solution and the hybridized cells were dried onto poly l-lysine coated quartz slides prior to Raman spectrum acquisition. Using the modified instrumentation and tube hybridization protocols we observed good resolution of Fluos-, Cy3- and Cy5-labelled cells (Fig. 2B) of our reference cultures as well as Fluos-labelled cells in complex naphthalene degrading samples (Fig. 2C, and see Determining substrate uptake in naphthalene degrading microcosms).

After demonstrations that FISH was compatible with imaging in air on the upgraded Raman microscope we evaluated the effect of different FISH fluorophores on Raman spectral resolution. Specifically, cells from each of three reference species (*Escherichia coli, P. fluorescens* and *Acinetobacter baylyi*) were hybridized in separate experiments with probes labelled with Fluos, Cy3 and Cy5 (Table 1). All species displayed highly similar results, and thus only the data relating to *P. fluorescens* are presented. No discernible fluorescence background in the Raman spectra was observed when using Fluos or Cy5 as the label for the FISH probe (Fig. 3A). However, since a-532.17 nm NdYAG laser was used to acquire the Raman spectra, large amounts of background fluorescence were observed when cells hybridized with Cy3-labelled probes were analysed (Fig. 3A). However, this problem could be solved by inclusion of a short bleaching step of 3 min in duration with the incident NdYAG laser prior to spectra acquisition. Time-course assays of this procedure (Fig. 3B) indicated rapid bleaching of the Cy3 label to reveal the underlying Raman spectrum, with a resolution comparable with those obtained for cells without the presence of a fluorophore.

**Calibrating Raman spectral shifts to cellular $^{13}$C-content in live and fixed/FISH hybridized cells**

In order to calibrate the $^{13}$C-labelling content of individual cells with Raman microspectroscopy peak shifts, the pronounced and strongly shifted phenylalanine peak was used as the determinative marker. Triplicate cultures of *E. coli, P. fluorescens* and *A. baylyi* were grown in media with increasing proportions of $^{13}$C-glucose as the sole source of carbon. Subsequently, 10–20 cells from each replicate sample were analysed as either fresh cells, or after fixation and hybridization with specific Fluos-labelled FISH probes (Table 1). Finally, the ratio of phenylalanine peak heights at 1003 cm$^{-1}$ (unlabelled) and

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target</th>
<th>Sequence 5′–3′</th>
<th>% FA a</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EUB338 Mix</td>
<td>Bacteria</td>
<td>GCTGCTTCCCCGTAGGAGT</td>
<td>30</td>
<td>Daims et al. (1999)</td>
</tr>
<tr>
<td>(EUB338 I, II &amp; III)</td>
<td></td>
<td>GGAGCCACCGCTAGGTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSM-G</td>
<td><em>Pseudomonas</em> spp.</td>
<td>CCT TCCTCCCAACTT</td>
<td>30</td>
<td>Braunhowland et al. (1993)</td>
</tr>
<tr>
<td>ACA652</td>
<td><em>Acinetobacter</em></td>
<td>ATCCCTTCCCCACCTCTA</td>
<td>35</td>
<td>Wagner et al. (1994)</td>
</tr>
<tr>
<td>ENT</td>
<td>Enterobacteriaceae</td>
<td>CCCCGCTTGGTGTGTCCTG</td>
<td>30</td>
<td>Kempf et al. (2000)</td>
</tr>
</tbody>
</table>

Probes, targets and hybridisation conditions were obtained from probeBase (Loy et al., 2003) and hybridisation and wash conditions used as described previously (Daims et al., 2006).

a. Percentage formamide in hybridisation.
b. Equimolar mix of all oligonucleotides.
967 cm\(^{-1}\) (labelled) (Fig. 4, inset) was calculated for all spectra obtained to yield a red shift ratio (RSR). Averaging these values over all species for each \(^{13}\)C-labelling level, indicated that the \(^{13}\)C-RSR fell between 0.5 for unlabelled cells and approximately 1.3 for fully labelled cells of the reference cultures (Fig. 4). Specifically, the relationship between RSR and \(^{13}\)C content of the cells for both live cells and fixed/FISH hybridized cells was linear and exhibited a high degree of correlation in both cases (\(R^2 = 0.98\) and 0.94 respectively). Analyses of both relationships indicated that the regression models derived were almost identical, and suggested that fixation and FISH hybridization prior to Raman analysis had negligible effects on the RSR-\(^{13}\)C content relationship, when compared with live cells. Furthermore, these data indicated that, subject to appropriate calibrations, the combination of FISH and Raman protocols provided a sound basis for the determination of \(^{13}\)C content within the probe delimited cells.

**Fig. 2.** Bright field and fluorescence micrographs of cell populations imaged in air using fluorescence augmented Raman equipment. A. Bright field image of a *Crenothrix polyspora* filament (Stoecker et al., 2006) after Raman analyses of two adjacent cells. High laser powers were used to burn the cells separately to demonstrate the sampling resolution of the confocal system (arrowed). B. Fluorescence micrographs of FISH hybridized cells imaged in air for *E. coli* labelled with ENT-Cy3 (Red), *P. fluorescens* labelled with PSM-G-Fluos (green) and *A. baylyi* labelled with ACA652-Cy5 (blue). C. Naphthalene degrading communities after incubation with 300 \(\mu\)m \(^{13}\)C10-labelled naphthalene and hybridization with EUBMIX (false coloured purple) and Fluos-labelled PSM-G to detect the presence of *Pseudomonas* spp. prior to Raman spectral acquisition. All scale bars represent 10 \(\mu\)m.

**Fig. 3.** A. Effects of FISH probe label on Raman single cell spectral quality for *P. fluorescens* when hybridized with EUBMix and labelled with either Fluos, Cy3 or Cy5. B. Laser induced bleaching of Cy3 background fluorescence during three sequential 60 s Raman spectral acquisitions for Cy3-labelled EUBMix probed *P. fluorescens*. © 2007 The Authors

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Positive PSM-G FISH signals were observed in the amended samples (Fig. 2C) and the phenylalanine RSR was compared for PSM-G detected cells in the $^{12}$C- and $^{13}$C-naphthalene incubations. For the $^{12}$C-control, PSM-G stained cells displayed a mean RSR value close to the baseline of 0.61 ± 0.07 ($n = 20$), whereas the mean RSR value for PSM-G positive cells in the $^{13}$C-incubations was 1.16 ± 0.04 ($n = 19$).

Finally, in a pure culture experiment we determined the correlation between the phenylalanine RSR and the degree of naphthalene-conferred $^{13}$C-labelling for one of the P. fluorescens isolates (Fig. 5 inset). Based on this calibration, and the assumption that assimilation of labelled naphthalene was similarly correlated with phenylalanine labelling for the FISH detected cells (as was observed for the pure culture calibrations), the RSR of each of the in situ detected Pseudomonas cells was converted into cellular $^{13}$C incorporation (atom%). The 19 measured cells were on average substantially labelled (approximately 50 atom%; dashed line, Fig. 5) confirming their uptake of the $^{13}$C-labelled naphthalene substrate within the groundwater microcosms. Interestingly, a pronounced heterogeneity of $^{13}$C-labelling was observed within the PSM-G positive population (Fig. 5). Specifically,

**Determining substrate uptake in naphthalene degrading microcosms**

In order to demonstrate single cell uptake of $^{13}$C-labelled compounds in probe identified bacteria in natural populations, we amended replicate samples of naphthalene degrading groundwater (sourced from a naphthalene contaminated gasworks site, Exeter, UK) with $^{13}$C10-labelled naphthalene for 72 h at 14°C. For $^{13}$C10-labelled naphthalene incubations we determined by flow cytometry the average total count to be 1513 cells ml$^{-1}$ (± 23). Subsequent plating of the incubations onto minimal medium containing naphthalene as a sole source of carbon yielded counts on average of 240 cells ml$^{-1}$, some 16% of the total population. Of these culturable naphthalene degraders, 58% were assigned to a P. fluorescens-like isolate by 16S rRNA sequencing (‘pre-INT2’; GenBank Accession number EF413073), and the remaining isolates could be split between two Pseudomonas putida-like isolates ‘pre-INT1’ (25% of the isolates, GenBank Accession number EF413072) and ‘pre-INT3’ (16% of the isolates, GenBank Accession number EF413074).

To test whether these bacteria were involved in naphthalene degradation in situ, Raman-FISH analysis was performed on $^{12}$C and $^{13}$C amended naphthalene groundwater incubations by using the Fluos-labelled Pseudomonas-specific probe PSM-G (Table 1) which displayed homology to all Pseudomonas isolates obtained.
derived \(^{13}\)C atom\% values ranged from 11\% (cell 2) to approximately 100 atom\% (cell 6), indicating naphthalene metabolism within the probe delimited population did not proceed at a uniform rate and, as such, the detected cells displayed a variable physiological response with respect to the degradation of the compound.

**Discussion**

The combination of FISH, the spectroscopic power of Raman microscopy and metabolic labelling through stable isotopes was developed as a new tool for single-cell structure–function analyses in complex microbial communities. The development of Raman-FISH is significant in two respects; as a complement to FISH-MAR (Lee et al., 1999), and as an augmentation to ‘whole community’ sequence recovery strategies such as stable isotope probing (Radajewski et al., 2000; Manefield et al., 2002a).

In relation to FISH-MAR, Raman-FISH provides comparable or higher resolution, has analysis times of approximately 1 min per cell, removes the dependence on radioactive isotopes and allows the quantification of tracer-induced signals. For SIP-based studies, Raman-FISH allows the analysis of recovered sequences at the single cell structure/function level within a single pulsing regime, as opposed to using a combined stable isotope/radioactive approach (Ginige et al., 2004).

In general, the successful development of Raman-FISH for the purpose of single-cell structure–function analyses principally depended on the merging of several characteristics. First, the fitting and optimization of epifluorescence capabilities to a commercially available Raman microscope to allow FISH analyses. Second, exploitation of recent instrument developments geared towards the confocal capabilities of Raman microspectroscopy (Nithipatikom et al., 2003). Finally, the ability to distinguish labelled cells from unlabelled cells after pulse labelling with stable isotopes, and the quantification of the isotope therein (Huang et al., 2004).

**FISH is compatible with Raman microspectroscopy of individual microbial cells**

In our strategy, we first evaluated whether the FISH protocol, including the fixation of the cells, disturbed or modified the Raman spectra of microorganisms. For this aspect of the development, probes labelled with one of the three most commonly applied FISH fluorophores (Fluos, Cy3 and Cy5) were used to specifically label reference cells prior to drying on the quartz slides required for Raman spectroscopic analyses (Manoharan et al., 1993). For Fluos and Cy5-labelled cells direct recording of Raman spectra was possible. As the wavelength of the applied Raman laser (532 nm) is well within the excitation range of Cy3, Cy3-labelled cells emitted a strong fluorescence band during Raman analysis, effectively masking the weaker Raman emission. However, this problem could be solved by including an additional bleaching step prior to Raman analysis. In addition, these analyses demonstrated that the whole FISH procedure including parafomaldehyde fixation and ethanol dehydration did not disturb Raman analysis of the microbial cells.

One issue regarding current Raman instrumentation in relation to FISH capabilities is that we found it necessary to fit an additional external camera for fluorescence imaging, in addition to the internal Raman microscope camera. This was principally due to the low sensitivity of current cameras used on Raman microscopes (generally linked to bright field detection of samples). However, as applications develop, we believe increased sensitivities of Raman microscope CCD detectors suitable for fluorescence capture (e.g. Cy5 fluorescence) and integrated epifluorescence imaging/Raman spectra capture software should allow the streamlining of this procedure onto a single camera interface.

**Determining the \(^{13}\)C-content of FISH-labelled microbial cells by Raman microspectroscopy**

We previously demonstrated red shift phenomena within spectra associated with \(^{13}\)C stable isotope-labelled cells (Huang et al., 2004). In the work described here, we developed this premise further for incrementally \(^{13}\)C-labelled cultures, using \(^{12}\)C-glucose or naphthalene as the sole carbon source in minimal media. In general, the major cellular constituents observed to red-shift were proteins and nucleic acids, both together making up significant components of the total cellular biomass (Neidhart, 1996). We also observed a unique and sharp spectral resonance for phenylalanine (Naumann, 2001) which exhibited a strong \(^{13}\)C-induced red shift, presumably due to substitution of the phenyl ring C atoms (Liu et al., 1990). Due to these strong spectral shifts and the fact that phenylalanine is an essential alpha amino acid present in all living cells we subsequently chose this region as the marker for calibration work involving the relative determination of \(^{13}\)C labelling in whole cells.

When analysing phenylalanine spectral shifts in response to \(^{13}\)C incorporation for different species we observed a strong relationship between the RSR and the \(^{13}\)C content, irrespective of the presence or absence of a FISH probe within the cell. This suggested that the spectral shift of the phenylalanine peak was not affected by FISH processing protocols and could be used as a basis to quantify the \(^{13}\)C-content of specifically probed microbial populations. In pure culture calibrations, we used a minimum labelling level of 10 atom\% \(^{13}\)C, a level which produced spectral shifts which could be reproducibly
detected from spectra which were only corrected for baseline. Therefore, there is now a requirement to assess the lower limit of significant spectral shifts (i.e. those generated at 13C labelling levels of less than 10% 13C), as this was considered outside the capabilities of this study. The reason for this was due to the nature of Raman spectra, in that accurate detection of small spectral shifts against a variable baseline more than likely requires specialist mathematical smoothing algorithms for spectral processing prior to spectral shift determinations, e.g. wavelet-based noise reduction (Wang et al., 2006). As such, it is likely that development of these spectral processing approaches should increase the precision of the method further. However, it must be noted that even if the sensitivity of Raman-FISH is not suitable below a labelling level of 10 atom% 13C, other popular methods which exploit stable isotope labelling have comparable or worse detection limits. For example, RNA-SIP requires a 13C-labelling of the RNA above 15% (Manefield et al., 2002b) for efficient separation of nucleic acids. Moreover, for DNA-SIP it has been proposed that the DNA 13C-content should be at least 50% to allow for a successful separation which is not biased by the varying GC-content of bacterial genomes. (Radajewski et al., 2003).

**Choices of macromolecules for determining labelling index and fractionation considerations**

We chose phenylalanine as a surrogate marker because it is highly visible and easily detectable in the many spectra analysed. Furthermore, because the red shift effect is presumably due to the sequential labelling of a well defined moiety, we suspect that similar relationships should broadly hold true for phenylalanine in a range of bacterial species. The main provisos are that phenylalanine can be detected within spectra and that isotopic labelling is passing through this central metabolite. If however, either or both of these factors are not the case there are many other spectral regions which were observed to be red-shifted after 13C isotopic labelling. As such, for cases where appreciable levels of phenylalanine are absent within spectra, or there is a diversion of metabolically labelled products to other pathways (e.g. storage compounds), the spectroscopic power of the Raman instrumentation could be used to resolve the labelling in other components of the cell after appropriate calibrations. Ultimately, this serves to increase the power and flexibility of Raman-FISH, i.e. the coupling of phylogenetic information, metabolic labelling and ultimately the assessment of the label passage through a range of cellular constituents. The power of this analysis is only limited by the resolving power of the microspectroscopy and appropriate spectral databases.

One assumption present in all studies which use cellular labelling to assess function is that fractionation between the heavier 13C and lighter 12C isotope is negligible. Isotopic fractionation has indeed been reported for specific organisms, albeit at natural abundance levels of 13C, e.g. methanotrophs (Templeton et al., 2006), photosynthetic organisms (O’Leary, 1984), toluene degraders (Meckenstock et al., 1999; Morasch et al., 2001) and for certain cell components in E. coli (Blair et al., 1985). However, it is still relatively unknown as to what degree the fractionation phenomenon may occur at higher 13C enrichments (e.g. such as those deployed during stable or radioactive labelled substrate pulsing regimes). For stable isotopes at least, we previously observed little or no fractionation against 13C when analysing key extracted biomarkers (Manefield et al., 2002b) after the same growth-based labelling of cells as was performed in these experiments. Nevertheless, we suggest that future applications could benefit from a parallel investigation of this effect using both the rapid Raman approach and the absolute quantification afforded by SIMS.

**Raman-FISH application in naphthalene degrading communities**

In order to demonstrate the applicability of the developed Raman-FISH method we designed a proof of principle experiment with a naphthalene degrading groundwater community as a model system. This system was chosen because selective cultivation on naphthalene medium had indicated that almost all culturable naphthalene degraders were affiliated with the genus *Pseudomonas*, and thus a simple experiment with a single specific FISH probe should be sufficient to prove the *in situ* activity of these naphthalene degraders by Raman-FISH. We incubated groundwater samples which are routinely exposed *in situ* to naphthalene and observed the phenylalanine RSR of *in situ* detected pseudomonads was much higher in the 13C10-labelled naphthalene incubation than in the 12C-naphtalene control. Subsequent calibration of these RSR values from a 13C-naphthalene pure culture experiment indicated that the pseudomonads detected *in situ* had an average 13C-content of approximately 50 atom% 13C.

Therefore, using the Raman-FISH approach, we could directly assign and quantify the metabolism of the labelled compound *in situ* to a key group which we knew to possess the capability from independent measures (selective isolation and molecular characterization of the strains). Such whole cell findings confirm previous reports on the naphthalene degrading capability of members of the genus *Pseudomonas* (Cane and Williams, 1982; Yen and Serdar, 1988; Simon et al., 1993; Stuart-Keil et al.,...
Experimental procedures

Standardization of Raman analyses using
\(^{13}\)C-glucose-labelled reference cultures

For initial testing and specific calibration of Raman signatures from \(^{13}\)C-labelled cells, a standard collection of incrementally \(^{13}\)C-labelled cells were produced for three reference cultures. In order to generate incremental levels of \(^{13}\)C labelling cells were grown in minimal medium where the ratio of \(^{13}\)C to \(^{12}\)C content of the sole carbon source was varied. Specifically, \(E. coli\) (K12), \(P. fluorescens\) (SBW25) and \(A. baylyi\) (ADP 1) were grown in M9 medium (Maniatis et al., 1989) with 5 g l\(^{-1}\) glucose as a sole source of carbon. The glucose added to the medium contained different amounts of \(^{13}\)C by mixing \(^{12}\)C \(\beta\)-glucose with 99 atom% \(^{13}\)C \(\beta\)-glucose (Sigma chemicals). Both unlabelled and labelled glucose was mixed in appropriate ratios (accounting for natural abundances of approximately 1% \(^{13}\)C in \(^{12}\)C glucose), to yield growth medium varying from 1 to 50% \(^{13}\)C in 10% increments, followed by 75% and 99 atom% as \(^{13}\)C. Furthermore, in order to account for possible mixing variance, three replicates of each medium were prepared and used to culture three biological replicates for each species. Cells were grown overnight with shaking at 37°C (E. coli) or 28°C (P. fluorescens and A. baylyi). Cells were subsequently pelleted at 14 000 \(g\), washed in phosphate-buffered saline (PBS), and either measured directly ('live' cells), or processed further for FISH ('Fixed/FISH hybridized cells') as detailed below.

Fixation and FISH prior to Raman analysis

Isolates (and natural samples, see below) were fixed prior to FISH by standard protocols (Daims et al., 2006). Briefly, cells were centrifuged at 14 000 \(g\), washed three times in PBS, fixed with three volumes of 4% paraformaldehyde in PBS and incubated on ice for 2 h. Subsequently, cells were washed twice in PBS prior to resuspension in a 1:1 (vol : vol) mix of PBS and 96% (vol : vol) ethanol, prior to storage at \(-20^\circ\)C.

For FISH, 50 \(\mu l\) of fixed samples were pelleted at 14 000 \(g\) for 10 min, resuspended in 100 \(\mu l\) 96% Analagar grade ethanol and incubated for 5 min at room temperature for dehydration. Subsequently, the samples were centrifuged at 14 000 \(g\) for 25 min, the ethanol was removed and the cell pellet was air dried. Cells were hybridized in solution (100 \(\mu l\)) for 2 h at 46°C. The hybridization buffer consisted of 900 mM NaCl, 20 mM TRIS HCl, 1 mM EDTA, 0.01% SDS and contained 100 ng of the respective fluorescently labelled oligonucleotide as well as the required formamide concentration to obtain stringent conditions (Table 1). After hybridization, samples were centrifuged at 14 000 \(g\) for 25 min, resuspended in wash buffer of appropriate stringency (Daims et al., 2006) and incubated for 15 min at 48°C. After washing, the cells were centrifuged for 15 min at 14 000 \(g\) and resuspended in 10 \(\mu l\) of PBS. One \(\mu l\) of the sample was spotted onto a poly l-lysine coated quartz slide (Agar Scientific; coating according to standard protocols of Daims et al., 2006). After this step, the slide was dipped for 2 s in ice-cold Milli-Q water and dried rapidly by compressed air from a microscope optics cleaning ‘mini-duster’ canister. Subsequent microscopic detection of the FISH signals and Raman analyses were performed without mounting media or coverslips.
Raman analyses

Raman microscopy was performed using a LabRAM HR800 UV confocal Raman microscope (Jobin-Yvon, UK) configured around an Olympus BX-41 microscope. The Olympus microscope chassis was modified with a 100-W Xenon lamp and standard FITC, CY3 and CY5 filter blocks and an F-View camera (Soft Imaging Systems) in consultation with the manufacturers (Jobin-Yvon, pers. com.). For combined Raman-FISH analyses, cells displaying oligonucleotide hybridization signals were located using epifluorescence imaging on the modified Raman. Once a cell was selected for Raman analysis, image acquisition was switched to the inbuilt Raman CCD detector. Every few hours, the alignment of the instrument was checked to compensate for drifts caused by machine vibration or heating of the optics. This was performed by imaging a fixed point on a slide through the Raman acquisition software. Subsequently, 1% of the incident laser light was allowed through the optical system to allow the imaging of the location of the laser beam area. Finally, the position of the beam area was compared to the position of the fixed analysis region within the acquisition software, and if required, the analysis region was manually moved to align it to the beam area.

For Raman spectral analysis of a chosen cell, the Raman scattering was excited by an NdYAG laser at 532.17 nm and the incident laser power was typically adjusted to around 5–8 mW, to avoid damaging of the sample while still maintaining spectral sensitivity. The pinhole of the Peltier cooled CCD detector (open electrode format) was set to 100 μm enabling a spatial resolution of approximately 1 μm (Huang et al., 2004). The system was checked for alignment prior to analyses using a silicon Raman reference (520 cm⁻¹) by acquiring a single spectrum from the silicon coated slide and ensuring the displayed data exhibited the required distribution and coefficient of variation around the 520 cm⁻¹ wave number. For cells, typical spectral characteristics were obtained by sequential acquisition and mathematical binning of two analyses of 30 s duration each. The Raman spectra were acquired between the range 2167.7 and 551.554 cm⁻¹ with 1022 data points encompassing this region (approximately 1.5 cm⁻¹ per point).

Raman spectra were processed for baseline correction and normalization using commercial LabSpec software (Jobin-Yvon). These data were then converted to ASCII files and exported to MatLab (The Maths Works) or Excel (Microsoft) for further peak determinations and calculations of the RSR.

Pulse labelling of groundwater with 13C-naphthalene, cell cultivation, counting and molecular analyses

Fully 13C-labelled naphthalene was purchased from Isotec and 14C-naphthalene from Sigma. Naphthalene was dissolved in dimethylformamide to make a 30-mM stock solution and 13C10-naphthalene or 12C10-naphthalene was introduced into replicate 250 ml of groundwater samples at a final concentration of 300 μM. Groundwater samples were obtained from an old gasworks site located in Exeter, UK. Briefly, site contamination comprised of priority organic contaminants in the soil and groundwater, which included polycyclic aromatic hydrocarbons (up to 1239 mg kg⁻¹ and 0.04 mg l⁻¹ respectively) of which naphthalene was the dominant chemical species (approximately 10% w/w). After incubation at 14°C (in situ groundwater temperature) for 72 h in the dark, bacterial cells from approximately 250 ml of groundwater were collected by filtration onto 0.22 μm Durapore filters (Millipore). Filters were subsequently vortexed with 10 ml of PBS in a 50-ml conical tube to detach cells, followed by centrifugation of the biomass at 5000 g for 10 min to recover the biomass. The biomass was subsequently resuspended in 500 μl of PBS, fixed, processed and hybridized as described above. In parallel, 10 μl aliquots of the incubations were plated onto 1.4% noble agar solidified minimal medium (Huang et al., 2005) and incubated upside down with a single naphthalene crystal in the lid of the Petri dish to provide the sole source of carbon. Plates were incubated at 14°C for 48 h in the dark and emerging colonies were streaked to purity on fresh plates and subsequently identified by denaturing gradient gel electrophoresis (DGGE) screening and 16S rRNA gene sequencing. For total cell counts, 300 μl of concentrated groundwater cell suspension was fixed with a 1% (w/v) final concentration of paraformaldehyde followed by nucleic acid staining with 0.3 μl of SYBR Green II (Molecular Probes, OR, USA) for 20 min in the dark. Samples were analysed using a FACSCalibur flow cytometer (Becton Dickinson) where samples were aspirated on a low flow rate (approximately 18 μl min⁻¹) and analysed with a 488-nm laser line. All samples were aspirated for a 3-min timed acquisition, the flow rates of the cytometer being calculated by weighing a standard sample before and after timed aspiration (20 min) to allow calculation of daily flow rate and subsequent absolute cell concentrations. Furthermore, internal standardization was performed by the addition of 2.49 μm Nile red beads (Becton Dickinson) to the sample at known concentrations to account for alignment and flow rate variation between samples.

For Raman-FISH calibrations in naphthalene degrading groundwater one of the isolates (P. fluorescens ‘pre-INT2’) was cultured on minimal liquid medium (Huang et al., 2005) containing 200 μM-naphthalene present in various mixing ratios to provide incremental 13C contents of 1 (natural abundance), 10, 25, 50 and 99 atom% 13C within the growth medium. Cells were grown overnight with shaking at 28°C and were subsequently pelleted at 14 000 g, washed in 500 μl of PBS and processed from Raman spectral acquisition as for ‘live’ cells detailed above.

Denaturing gradient gel electrophoresis screening of isolates and 16S rRNA gene sequencing

For characterization of the isolates by 16S rRNA molecular analyses, DNA from the isolates obtained by selective plating were extracted and subject to DGGE screening prior to almost full length 16S rRNA sequencing according to previous protocols (Whiteley and Bailey, 2000). Short (200 bp) DGGE polymerase chain reaction (PCR) products were separated on 10% (wt/vol) polyacrylamide gels with a 35–70% urea/formamide denaturing gradient at 60°C and 100 V for 16 h. Gels were stained with SYBR gold (Molecular Probes) and visualized by UV trans-illumination. Subsequently, isolates were grouped based on unique banding patterns and representatives selected for near full length 16S
rRNA sequencing. Briefly, almost full length 16S rRNA genes were PCR-amplified using primers 63f and 1387r (Marchesi et al., 1998), the PCR products were subsequently purified and direct sequencing of the amplicons was performed with Big Dye chemistry and an ABI 3700 sequencer according to the manufacturer's instructions (Applied Biosystems). The 16S rRNA gene sequences obtained were phylogenetically analysed using the ARB software package for placement (Ludwig et al., 2004) and, when required, 16S rRNA probes and hybridization information were downloaded from probeBase (Loy et al., 2003).

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