ORIGINAL ARTICLE

Protein-based stable isotope probing (Protein-SIP) reveals active species within anoxic mixed cultures

Nico Jehmlich¹, Frank Schmidt¹, Martin von Bergen¹, Hans-Hermann Richnow² and Carsten Vogt²

¹Helmholtz—Centre for Environmental Research—UFZ, Department of Proteomics, Leipzig, Germany and ²Helmholtz—Centre for Environmental Research—UFZ, Department of Isotope Biogeochemistry, Leipzig, Germany

It is still a challenge to link specific metabolic activities to certain species in a microbial community because of methodological limitations. We developed a method to analyze the specific metabolic activity of a single bacterial species within a consortium making use of [¹³C₇]-toluene for metabolic labelling of proteins. Labelled proteins were subsequently analyzed by 2D gel electrophoresis (2-DE) and mass spectrometry (MS) to characterize their identity as well as their ¹³C content as an indicator for function and activity of the host organism. To establish this method, we analyzed the metabolic incorporation of ¹³C carbon atoms into proteins of *Aromatoleum aromaticum* strain EbN1. This strain is capable of metabolizing toluene under nitrate-reducing conditions and was grown in either pure culture or in a mixed consortium with a gluconate-consuming enrichment culture. First, strain EbN1 was grown with non-labelled toluene or labelled [¹³C₇]-toluene as carbon sources, respectively, and their proteins were subjected to 2-DE. In total, 60 unique proteins were identified by MALDI-MS/MS. From 38 proteins, the levels of ¹³C incorporation were determined as $92.3 \pm 0.8\%$. Subsequently, we mixed strain EbN1 and the enrichment culture UFZ-1, which does not grow on toluene but on gluconate, and added non-labelled toluene, [¹³C₇]-toluene and/or non-labelled gluconate as carbon sources. The isotope labelling of proteins was analyzed after 2-DE by MS as a quantitative indicator for metabolic transformation of isotopic-labelled toluene by the active species of the consortium. Incorporation of ¹³C was exclusively found in proteins from strain EbN1 at a content of $82.6 \pm 2.3\%$, as an average calculated from 19 proteins, demonstrating the suitability of the method used to identify metabolic active species with specific properties within a mixed culture.

The ISME Journal (2008) 2, 1122–1133; doi:10.1038/ismej.2008.64; published online 19 June 2008

Subject Category: integrated genomics and post-genomics approaches in microbial ecology

Keywords: *Aromatoleum aromaticum* strain EbN1; [¹³C₇]-toluene; metabolic labelling; stable isotope probing (SIP)

Introduction

Natural element cycles are basically driven by microbial communities typically consisting of several different species performing a variety of different biogeochemical reactions. For understanding the functionality of microbial communities, it is necessary to elucidate the role of individual species within a community. The traditional concept of isolation and subsequent characterization of the individual microorganisms (for a review, see Koch, 1881) is not appropriate for analyzing structure,

function and activity of microbial communities because of the fact that most prokaryotic species are not cultivable using traditional cultivation methods (Pace 1997; Hugenholtz *et al.*, 1998; Rappe and Giovannoni, 2003; Keller and Zengler, 2004). In addition, it was shown that often no single species but a microbial consortium containing several species could be isolated, which were dependent on their interaction during growth (Jimenez *et al.*, 1991; Pelz *et al.*, 1999; Drzyzga *et al.*, 2002; Barreiros *et al.*, 2003). Thus, for tracking the microbial-mediated flow of carbon and nitrogen in the environment, specific tracer techniques have to be developed. ¹³C-labelled compounds have been used to trace the transformation of compounds in slow-growing anaerobic microbial cultures degrading, for example, alkanes and chlorobenzene (Zengler *et al.*, 1999; Nijenhuis *et al.*, 2007).

Correspondence: Martin von Bergen, Helmholtz—Centre for Environmental Research—UFZ, Department of Proteomics, Permoserstrasse 15, Leipzig D-04318, Germany.

E-mail: martin.vonbergen@ufz.de

Received 24 January 2008; revised 20 May 2008; accepted 27 May 2008; published online 19 June 2008

Recently, stable isotope probing (SIP) techniques were developed employing the incorporation of isotopically labelled substrates (^{13}C or ^{15}N) into nucleic acids for detecting functional relationships within a microbial community. The metabolically active species assimilate the labelled substrate, which can then be separated from the non-labelled biomass by ultracentrifugation. By this approach, the metabolically active species can be identified using DNA (Friedrich, 2006) or RNA (Manefield *et al.*, 2002; Whiteley *et al.*, 2006) as target compounds. The drawbacks of this technique are the low resolution of the labelled and non-labelled DNA/RNA during ultracentrifugation and thereby the difficulty to quantify the uptake of the stable isotopes into DNA/RNA. About 30 atom % of ^{13}C incorporation is required for separation of labelled and non-labelled nucleic acids under experimental conditions (Tillman Lüders, personal communication). Moreover, the concentration of DNA/RNA is low compared to other cell constituents such as lipids and proteins. Despite the low resolution of SIP approaches based on DNA or RNA, they provide both phylogenetic information and a measure for metabolic activity (Friedrich 2006; Webster *et al.*, 2006). Therefore, detection of labelled functional genes is possible (Hutchens *et al.*, 2004; Lin *et al.*, 2004).

Stable isotope labelling of lipids (Richnow *et al.*, 2002; Geyer *et al.*, 2005; Webster *et al.*, 2006) or amino acids upon metabolism of isotopically labelled substances (Richnow *et al.*, 2002; Miltner *et al.*, 2005; Krüger *et al.*, 2008) is another concept to prove the transformation of organic compounds into the biomass. ^{13}C -labelled substrates in combination with ^{13}C -enrichment of phospholipid fatty acids (PLFA) were used to characterize bacterial toluene degradation in soil, sediment or aquifer microcosms (Hanson *et al.*, 1999; Pelz *et al.*, 2001a,b). The analysis of lipids in labelling experiments have been successfully applied to obtain information of methane-oxidizing communities in soils and sediments (Boschker *et al.*, 1998; Bull *et al.*, 2000). However, the most important question regarding which metabolic species is active, and their position in the food chain cannot be answered by this concept in many cases owing to the low phylogenetic value of lipids or free amino acids obtained after total hydrolyzation of proteins.

The most direct link to a molecular function is given by identification of proteins involved in the metabolic process of interest, as proteins actually catalyze the biochemical reactions. Thus, proteins provide phylogenetic and functional information, which make them ideal molecules for studying the structure and function of microbial communities. In the past years, the technical resolution and sensitivity of the mass spectrometer has been improved and the amount of genomic information increased significantly. Consequently, in recent studies, bacterial communities have been characterized by

proteomic approaches (Wilmes and Bond, 2006a). The investigated environmental samples showed an intermediate complexity and were analyzed either by a separation step using 2-DE (Wilmes and Bond, 2006b; Benndorf *et al.*, 2007) or by shotgun approaches (Ram *et al.*, 2005; Lo *et al.*, 2007). These studies revealed significant details about the analyzed communities, especially the different proteins involved in functional aspects.

The isotope labelling of proteins can be used as a combined indicator for a specific metabolic activity as well as for obtaining phylogenetic information. Metabolic or chemical incorporation of stable isotopes is widely used for quantitative proteomics (for a review, see Ong and Mann, 2005), but stable isotopes have rarely been used on a proteomic level as a tracer to prove the metabolism of organic substrates. The metabolic incorporation of stable isotope-labelled substrate into proteins has been used to elucidate the carbon metabolism of the hyperthermophilic *Sulfolobus solfataricus* (Snijders *et al.*, 2006) using ^{15}N -containing medium, which was compared to a parallel culture grown on ^{14}N medium. In another approach, the level of metabolic incorporation was used to determine half-life times of proteins by analyzing the incorporation of [$^{13}\text{C}_6$]-glucose in heat-shocked HeLa cells or of $^{15}\text{NH}_4^+$ into bacterial proteins in labelling experiments (Cargile *et al.*, 2004; Snijders *et al.*, 2005a).

The most important advantage of protein analysis is its direct connection to the physiological function of interest, for example, the metabolic degradation of xenobiotics. To examine the potential of protein stable isotope probing (protein-SIP), we conducted a model experiment for identifying the species responsible for anoxic toluene degradation in an artificial mixed culture fed with gluconate and [$^{13}\text{C}_7$]-toluene under denitrifying conditions. The mass spectrometric analysis of the proteomes following 2-DE enabled us to identify the active degrading species in a bacterial consortium.

Materials and methods

Chemicals

All chemicals used were of pro analysis quality and were purchased from Sigma (Munich, Germany) or Merck (Darmstadt, Germany). [$^{13}\text{C}_7$]-toluene (≥ 99 atom % ^{13}C) was obtained from Sigma-Aldrich (Germany). Non-labelled glucose and toluene have a natural isotope composition of about 1.075–1.076 atom % ^{13}C , respectively. Toluene with natural isotopic abundance is referred to as ^{12}C toluene.

Culture of bacteria

Aromatoleum aromaticum strain EbN1 was kindly provided by Matthias Boll, University of Leipzig, Germany. Cells were cultivated in a mineral salt medium as described elsewhere (Rabus and Widdel,

1995), spiked with toluene (0.57 mM) as the sole source of carbon and energy and nitrate (10 mM) as electron acceptor. Toluene was added as a pure compound by means of sterile syringes (Hamilton, Bonaduz, Switzerland).

The enrichment culture UFZ-1 was set up using 1 ml sludge taken from a pond located at the area of the Helmholtz Centre for Environmental Research—UFZ, Leipzig. The sludge was diluted in 50 ml modified and anoxic Brunner mineral salt medium (Deutsche Sammlung für Mikroorganismen, DSMZ, medium 457; modifications: use of 1 ml l^{-1} trace element solution SL-10 instead of SL-4; plus 5 ml l^{-1} vitamin solution (Pfennig *et al.*, 1965)) containing nitrate (20 mM) as the electron acceptor and gluconate (5 mM) as the sole source of carbon and energy. The enrichment culture grew within 48 h. Subsequently, the culture was transferred (2%) two times per week in mineral salt medium used for strain EbN1, containing gluconate (1–5 mM) as the carbon and energy source and 10–20 mM nitrate as the electron acceptor.

Degradation and growth experiments were carried out as fed batch cultures in tubes (20 ml), or serum bottles (56 or 118 ml volume, Glasgerätebau Ochs, Bovenden Lengern, Germany) filled with 15–80 ml culture suspension and closed with aluminium-crimped Teflon-coated butyl septa (ESWE Analysentechnik, Gera, Germany). Cultures were inoculated (5–10%) using cells in the early stationary growth phase, grown either on toluene (EbN1) or gluconate (UFZ-1) as sole source of carbon and energy under denitrifying conditions.

All used solutions were sterilized by filtration or autoclaving and flushed with N_2 (to remove oxygen) before use. Bacterial cultures were incubated statically at 25–30 °C in the dark. Inocula were transferred by means of nitrogen-flushed sterile plastic syringes (Hungate-technique), as samples for chemical and microbiological analyses were taken.

Cell growth was followed by determining changes in optical density at 600 nm (Spectrophotometer type: Novaspec II, GE Healthcare, Uppsala, Sweden) using either 20 ml reagent tubes directly for measurement or plastic cells filled with 1 ml bacterial culture against a blank made of uninoculated mineral salt medium.

Determination of toluene concentrations

Toluene was analyzed by automated headspace gas chromatography as described elsewhere (Fischer *et al.*, 2008).

Sample preparation for 2-DE electrophoresis

After cultivation, cells were harvested by centrifugation for 10 min at $15\,500 \text{ g}$ (Laboratory Centrifuge 3K30, Sigma, Osterode, Germany). Cell pellets were treated with protein inhibitor PMSF, lysed and

processed as described previously (Benndorf *et al.*, 2007, 2008). The protein concentration was determined according to the Bradford assay (Bradford 1976).

For 2-DE analysis, $200 \mu\text{g}$ of protein were precipitated with five-fold ice-cold acetone and the resulting protein pellet was air-dried and dissolved in DeStreak rehydration solution with 0.5% IPG (immobilized pH gradient) pH 3–10 non-linear (NL) buffer (v/v) (GE Healthcare, Uppsala, Sweden). To remove precipitates, the solution was centrifuged for 15 min at $62\,000 \text{ g}$. The equilibration was performed with $135 \mu\text{l}$ of supernatant loading on 7 cm Immobiline DryStrip pH 3–10 NL (GE Healthcare, Uppsala, Sweden) as described in more details in Georgiera *et al.* (2008). In the first dimension, proteins were focused on using IPG Phor electrophoresis unit overnight (GE Healthcare). Subsequently, the strips were placed in equilibration buffer containing 50 mM Tris/HCL pH 8.8, 30% glycerol (v/v), 6 M urea, 4% sodium dodecyl sulfate and 2% dithioerythrol as well as 2.5% iodoacetamide for 15 min. The second dimension was performed with SDS-polyacrylamide gel electrophoresis (PAGE) (Schagger *et al.*, 1988). SDS-PAGE was performed using the Laemmli-buffer system described elsewhere (Santos *et al.*, 2007), with an acrylamide concentration in the separating gel of 12%. After SDS-PAGE separation, proteins were visualized by colloidal Coomassie Brilliant Blue G-250 (CBB) (Neuhoff *et al.*, 1988) (Roth, Kassel, Germany) staining.

Sample preparation and MALDI-MS

Excised gel spots were cut from polyacrylamide gels and washed two times with methanol/acetic acid (50%/5%, v/v). Subsequently, the gel spots were digested overnight at 37 °C using trypsin (Sigma, Munich, Germany), according to Shevchenko *et al.* (1996), with minor modifications described elsewhere (Hehemann *et al.*, 2008). The resulting mixture of peptides was extracted two times with acetonitril/85% formic acid (50%/5.8%, v/v). The extracts were directly concentrated by vacuum centrifugation. Thereafter, tryptic peptides were acidified by adding 0.1% trifluoroacetic acid (TFA) (v/v) in ultra pure water.

MALDI-MS analysis was performed on a TOF/TOF instrument (Ultraflex III TOF/TOF mass spectrometer, Bruker Daltonics, Bremen, Germany). The measurements were performed in positive-ion reflector mode and externally calibrated. All samples were prepared on MTP GroundSteel target plates with transponder technology (Bruker Daltonics) by using α -Cyano-4-hydroxycinnamic acid (CHCA) dissolved in acetonitrile:TFA (50%/0.1%, v/v) as matrix. The peak selection was defined to use masses from 700 to 3000 Dalton for evaluation and processing. The peptide mass fingerprint (PMF) spectra were acquired by summing up 2000 laser

shots and tandem mass spectra (MS/MS-spectra) by summing up 1000 shots for the parent masses and 2000 shots for the fragment masses. Subsequently, the peak detections were calculated using flexAnalysis Version 3.0 (Build 54, Bruker Daltonics, Bremen, Germany). Therefore, an S/N of three and baseline subtraction were applied. Mass spectrometry (MS) and MS/MS peptide identifications were achieved by database comparisons (NCBI database, 20080403, all entries) using BioTools 3.0 (Bruker Daltonics, Bremen, Germany) in conjunction with the Mascot in-house version 2.2.1 (Matrix Science, London, UK). A tolerance of 100 p.p.m. was used for the precursor mass and 0.8 Da for MS/MS fragments. Furthermore, trypsin was selected as enzyme considering up to one missed cleavage site, and variable protein modifications were allowed, such as Met-oxidation and Carbamidomethyl of C.

Calculation of incorporation rates

For calculation of incorporation efficiency, we used the definition given by Snijders *et al.* (2005b). In addition, a Perl script provided with the supporting material to the study of Snijders *et al.* (2006) was used. In brief, this means that for a given isotopomer the incorporation efficiency is defined by the percentage of incorporated ^{13}C -atoms in relation to the total number of carbon atoms with the natural isotope abundance (about 1.01 atom % ^{13}C). For reference, we used the theoretical isotopic distribution as calculated by the software tool Isotope Pattern (Bruker Daltonics). For convenience, an Excel spreadsheet (ProSIPQuant.xls, see supplement) was created where the peak lists from selected peptides could be pasted. The Excel spreadsheet calculates the input for the first command line of the Perl script. Currently, no modifications like carboxymethyl (cysteine) or oxidation (methionine) on identified peptides were included in the calculation of incorporation by the Perl script. Therefore, only peptides without modifications were used.

Results

Growth pattern of *Aromatoleum aromaticum* strain EbN1 and the enrichment culture UFZ-1

To demonstrate the identification of proteins containing metabolically incorporated ^{13}C -atoms in a mixed culture, we first tested the stability of strain EbN1 and of the enrichment culture UFZ-1 in separate experiments. Strain EbN1 can grow under anoxic conditions with toluene, using nitrate as an electron acceptor (Rabus and Widdel, 1995), and we tested whether there was a difference in growth on [$^{13}\text{C}_7$]-toluene compared with non-labelled toluene. As shown in Figure 1a, growth curves for non-labelled and labelled toluene were similar, demonstrating that strain EbN1 did not discriminate significantly between ^{12}C and ^{13}C . With the increase

of biomass, the toluene concentration decreased concomitantly and the given 0.5 mM were almost entirely consumed after 20 h. During this time period, the culture also reached the stationary phase.

The enrichment culture UFZ-1 can grow on gluconate but not on toluene (Figure 1b). Using gluconate (2 mM) as the sole source of carbon and energy, UFZ-1 reached the stationary phase after approximately 10 h. The mixed nutrition by gluconate and toluene did not alter the growth curve in comparison to the growth on gluconate as sole carbon source (Figure 1b), showing that the given concentration of toluene was not toxic for UFZ-1. We chose gluconate as a carbon source for the culture UFZ-1, as strain EbN1 lacks all necessary genes for the degradation of this compound (Rabus and Widdel, 1995). Thus, strain EbN1 cannot grow with gluconate as the sole source of carbon and energy (Figure 1c), as also tested in growth experiments in our laboratory before performing experiments with artificial mixed cultures (data not shown). Gluconate acted, therefore, as a substrate that allows growth of the enrichment culture UFZ-1, but not of strain EbN1. Single and mixed cultures reached similar optical densities when only one substrate was added. Significantly higher optical densities were reached when both substrates were present (Figure 1c).

Separation on 2-DE

In the next step, we established the method of analysis for metabolic labelling of strain EbN1 by growing on ^{12}C - and [$^{13}\text{C}_7$]-toluene by subsequent 2-DE and MS analysis. The 2-DE from samples of strain EbN1 grown on ^{12}C -toluene or ^{13}C -toluene, respectively, showed a high accordance after visualization by Coomassie staining (data not shown). From this gel, 60 spots were picked, and the proteins were unambiguously identified by peptide mass fingerprint and MS/MS-analysis after tryptic digestion (Supplementary Table 1a). For all, at least four peptides were found and the mean of the sequence coverage of all of them was approximately 48%. For the experiment with EbN1 growing on [$^{13}\text{C}_7$]-toluene (Figure 2a), we expected a mass shift of the proteins in the second dimension of the 2-DE electrophoresis. A complete labelling of the proteins with ^{13}C -atoms would account theoretically for an increase in mass of about 5%, which we actually cannot unambiguously detect in the gels due to dependency of the electrophoretic mobility on the ratio charge/length that remains unaffected in case of labelling with stable isotopes. The incorporation of ^{13}C -atoms into proteins from the strain EbN1 did not hamper the general appearance of the spots on the gel, which enabled a comparison between distinct spots from the gels made of samples from the non-labelled and [$^{13}\text{C}_7$]-labelled pure culture of EbN1 and of those from the mixed

culture consisting of EbN1 and UFZ-1 (Supplementary Tables 1b and c).

Mass spectrometry of control samples grown on natural ^{12}C -toluene allowed identification of proteins and enabled determination of incorporation levels

From the identified proteins, we looked in more detail at the PMF spectra and found a slightly increased isotopic noise in the ^{13}C -sample, consisting of many isotopic peaks around the most abundant isotopomers (Figures 3a and b). We calculated the incorporation rate in the ^{13}C -sample by using peaks that were used in the PMF analysis for identification of the ^{12}C -sample of the corresponding protein. In the case of peak m/z 1611.79 (SLGQFNLSIPPAPR) (Figure 3c) from the chaperone protein dnaK (EbN1), we picked the highest peak of the ^{13}C -sample at m/z 1678.83 (Figure 3d) and determined an incorporation level by an error minimization approach (Snijders *et al.*, 2005b) of 92%. This approach was used because of the complex pattern where no real monoisotopic mass could be defined. The same procedure was applied to at least three peptides of 38 proteins and the incorporation levels resulted in a mean value of 92.3% with a relative s.d. of 0.8% (Supplementary Table 2a). The details of the isotopic distribution also revealed the history of the culture. In the case of the peptide SLGQFNLSIPPAPR (protein DnaK), there was still a peak observable from non-labelled peptide containing almost exclusively carbon atoms of natural composition, which must stem from cells of the inoculum that were not metabolically active in the presence of $^{13}\text{C}_7$ -toluene.

MS/MS-analysis revealed details of ^{13}C -incorporation in amino acids

On the basis of the analysis on the peptide level in the PMF spectra, only the average of incorpora-

tion of ^{13}C could be calculated. The mode of ^{13}C -incorporation became obvious after fragmentation of the peptides by MS/MS-analysis. The peptide SLGQFNLSIPPAPR at m/z 1611.79 showed a regular fragmentation in the ^{12}C -sample (Figure 3e) as well as the precursor of m/z 1678.83 from the ^{13}C -sample (Figure 3f). The y_1 -ions of arginine were clearly visible in the low molecular weight part of the spectra (insets in Figures 3e and f). The y_1 -ion of arginine contains six carbon atoms

Figure 1 Growth of strain EbN1, the enrichment culture UFZ-1 and an artificial mixed culture composed of EbN1 and UFZ-1 with different substrates under denitrifying conditions. (a) Growth of strain EbN1 in the presence of 10 mM nitrate with ^{13}C labelled (open circles) and non-labelled (open triangles) toluene determined by the increase of OD_{600} and the decrease of toluene concentrations (labeled—filled circles; non-labelled—filled triangles). The development of the culture in the absence of toluene is shown by the dashed line. (b) Growth of the enrichment culture UFZ-1 with gluconate (open diamonds; 2 mM), toluene (filled triangles; 0.57 mM), gluconate (2 mM) + toluene (0.57 mM) (filled diamonds) or no substrate (grey circles) in the presence of 10 mM nitrate. (c) Growth of EbN1, UFZ-1, or mixtures of EbN1 and UFZ-1 with toluene (labelled or non-labelled; 0.57 mM), gluconate (1 mM), or mixtures of toluene (0.57 mM) and gluconate (1 mM) under denitrifying conditions (20 mM nitrate). Shown is the optical density (OD_{600}) of stationary phase cultures after 5 days incubation: bar (a), EbN1, no substrate; (b) UFZ-1, no substrate; (c) EbN1 + UFZ-1, no substrate; (d) EbN1, ^{12}C -toluene; (e) EbN1, $^{13}\text{C}_7$ -toluene; (f) UFZ-1, gluconate; (g) EbN1 + UFZ-1, gluconate; (h) EbN1 + UFZ-1, ^{12}C -toluene; (i) EbN1 + UFZ-1, $^{13}\text{C}_7$ -toluene; (j) EbN1 + UFZ-1, ^{12}C -toluene/gluconate; (k) EbN1 + UFZ-1, $^{13}\text{C}_7$ -toluene/gluconate.

Figure 2 2-DE separation of proteins from different culture samples. **(a)** Strain EbN1 grown on ^{13}C -toluene. Proteins marked A-01–A-60 were identified in the corresponding 2-DE from strain EbN1 fed on ^{12}C -toluene. **(b)** EbN1 and UFZ-1 grown on ^{13}C -toluene + gluconate. Proteins were identified in the corresponding 2-DE from the mixed culture fed on ^{12}C -toluene + gluconate, the identified proteins are annotated with B-01–B-40. The identified proteins from the enrichment culture UFZ-1 are marked C-01–C-40.

and has an m/z of 175.12 $[\text{M} + 1\text{H}]^+$. In the case of a complete incorporation of ^{13}C , the mass should be increased to m/z 181.14.

For the ^{12}C -sample, we found a peak at m/z 175.10, which agrees fairly with the nominal mass, but for the ^{13}C -sample the most intense peak was at m/z 181.38, indicating a complete labelling of arginine by six ^{13}C -atoms.

Mass spectrometry of a mixed culture of strain EbN1 and enrichment culture UFZ-1 grown on ^{13}C -toluene and non-labelled gluconate revealed ^{13}C -incorporation exclusively in proteins from strain EbN1

After establishing culture conditions, separation by 2-DE (Figure 2b) and analysis parameters for MS, we incubated strain EbN1 and the enrichment culture UFZ-1 as an artificial mixed culture for 5 days under denitrifying conditions (20 mM nitrate) with toluene (^{13}C -atom or ^{12}C -atom; 0.57 mM) and/or non-labelled gluconate (1 mM) as carbon sources, respectively. For comparison, single cultures of UFZ-1 and EbN1 were again set up. All gluconate and/or toluene-spiked cultures grew, as shown in Figure 1c. Toluene was completely consumed in each toluene-spiked microcosm, as revealed by GC-analysis. The highest optical densities were reached by mixed cultures fed with gluconate and labelled or non-labelled toluene (Figure 1c), demonstrating that besides toluene, gluconate was also metabolized. The single cultures, as well as the mixed culture, showed no growth without addition of substrate (Figure 1c).

The mass spectrometry analysis of proteins from EbN1 cells grown in this mixed culture revealed a slightly lower incorporation of ^{13}C ($82.6 \pm 2.3\%$; Supplementary Table 2b), compared with the single culture experiment ($92.3 \pm 0.8\%$; Supplementary Table 2a). The PMF spectra of chaperone protein dnaK of strain EbN1 showed a slightly increased isotopic noise (Figures 4a and c). From these spectra, several peptide masses were used for identification, first on the peptide and then by MS/MS analysis. The level of ^{13}C incorporation was calculated by comparing the spectra of the ^{12}C - and ^{13}C -sample. To show an example, the detailed analysis of the peptide SLGQFNLSDIPPAPR, m/z 1611.81 from chaperone protein dnaK (EbN1), is displayed in Figure 4e. The isotopic distribution of the non-labelled peptide (Figure 4e, upper part) followed nearly perfectly the theoretical isotopic distribution, whereas the isotopic distribution in the ^{13}C -sample is indicated by the shift of the most abundant isotopomer to a mass of m/z 1671.85 accompanied by a completely differently shaped isotopic envelope (Figure 4e, bottom part). For this peptide, an incorporation level of 83% was determined according to the scheme described in the Materials and methods section. We analyzed two more peptides from chaperone protein dnaK and found similar incorporation levels ranging from 83 to 86%. The average incorporation was 82.6%, with a deviation of $\pm 2.3\%$ (Supplementary Table 2a). In contrast, proteins not related to strain EbN1 and hence stemming from species of the UFZ-1 consortium were not enriched in ^{13}C -atoms after growth on ^{13}C -labelled toluene and gluconate. As an example, a heat-shock protein of the HSP20 family protein with a sequence homology to a gene of *Pseudomonas stutzeri* A1501 is shown (Figures 4b and d). The PMF spectra of samples grown on either

Figure 3 Mass spectrometry analysis showing peptide mass fingerprints and tandem mass spectra from proteins of EbN1 cultures fed on ^{12}C -toluene or $^{13}\text{C}_7$ toluene. Shown are the PMF-spectra of the chaperone protein dnaK (EbN1) cultured in the presence of (a) ^{12}C -toluene or (b) $^{13}\text{C}_7$ -toluene. The optical zoom shows in more detail the isotopic distribution of the peptide (SLGQFNLSDIPPAPR, m/z 1611.79) from the ^{12}C -toluene (c) and the $^{13}\text{C}_7$ -toluene (d) sample (m/z 1678.83). The MS/MS-analysis of this peptide is shown for the ^{12}C -toluene (e) sample and for the $^{13}\text{C}_7$ -toluene (f) sample. The optical insets in (e) and (f) show in more detail the isotopic distribution of the y_1 -ion of arginine.

^{12}C -toluene/gluconate or ^{13}C -toluene/gluconate exhibited nearly the same most abundant peaks and isotopic details of these peaks (Figure 4f, upper

and bottom part), respectively. These data confirm that ^{13}C -atoms were incorporated only in proteins related to strain EbN1.

Figure 4 Mass spectrometry analysis of selected proteins from the artificial mixed culture composed of EbN1 and UFZ-1 grown on different substrates. (a) The PMF-spectra of the chaperone protein dnaK (EbN1) from the mixed culture grown with $^{12}\text{C}_7$ -toluene/gluconate as well as $^{13}\text{C}_7$ -toluene/gluconate (c). (b) The PMF-spectra of the heat-shock protein, HSP20 family (gene sequence homology to *Pseudomonas stutzeri* A1501) from the mixed culture grown with $^{12}\text{C}_7$ -toluene/gluconate are displayed as well as the sample from a culture grown on $^{13}\text{C}_7$ -toluene/gluconate (d). (e and f) Optical zoom shows the ^{13}C incorporation into the peptide of EbN1 (SLGQFNLSDIPPAPR, m/z 1611.81), whereas no ^{13}C was incorporated in peptides belonging to the enrichment culture UFZ-1.

Discussion

Incorporation of carbon derived from the metabolization of [$^{13}\text{C}_7$]-toluene in proteins of strain EbN1

Strain EbN1 can grow on a wide range of aromatic compounds under denitrifying conditions, including toluene (Rabus *et al.*, 2005). The initial attack of toluene is catalyzed by the enzyme benzylsuccinate synthase (BSS), which adds fumarate to the methyl group of toluene, forming benzylsuccinate as the first metabolite of the degradation pathway (Biegert *et al.*, 1996). This step is well documented for several toluene-degrading pure cultures under anoxic conditions and seems to be a unique reaction for the activation of aromatic and aliphatic hydrocarbons without the help of oxygen. Benzylsuccinate is further transformed, catalyzed by five enzymes, to benzoyl-coenzyme A (benzoyl-CoA), which is a central metabolite for many anaerobic aromatic degradation pathways (for a review, see Widdel and Rabus, 2001). Benzoyl-CoA can be reduced and ring-cleaved by different mechanisms in subsequent metabolic steps (Boll *et al.*, 1997). We used strain EbN1 as a model organism for the specific detection of labelled proteins formed by the assimilation of toluene, in pure culture and artificially mixed cultures, using [$^{13}\text{C}_7$]-toluene as a tracer substance. We chose strain EbN1 as its genome has been recently sequenced (Rabus, 2005), hence allowing identification of proteins of strain EbN1 out of a mixture of different proteins with high reliability. The central enzyme for anoxic degradation of toluene, the benzoyl-CoA reductase, was found in the EbN1-culture, and the isotope incorporation was very similar to that of other proteins.

The growth yield of strain EbN1 on [$^{13}\text{C}_7$]-toluene under denitrifying conditions was the same as observed with ^{12}C -toluene as substrate (Figure 1), demonstrating that the cells did not significantly discriminate between both isotopes, which is a prerequisite for further applications of the method and further ecological studies. A high degree of incorporation of ^{13}C -atoms in proteins ($\sim 92\%$) was found under the given growth conditions.

On the basis of the increase in optical densities from about 0.02 to about 0.1–0.15 OD_{600} in the ^{13}C experiments with strain EbN1 (Figures 1a and c), cells divided approximately 2–3 times. In theory, this should cause a dilution of the present ^{12}C carbon down to 12–25%, leading to incorporation rates of 75–88%. The experimental value of 88–94% is significantly higher, indicating that other factors were involved. It is likely that proteins were also degraded and newly synthesized within a single growth cycle, hence leading to higher incorporation levels than estimated by a calculation based only on dilution. The relationship of cell growth and incorporation of ^{13}C -carbon in

proteins will be investigated in more detail in further studies.

Growth of an artificial mixed culture on gluconate and toluene and proof of toluene metabolization by strain EbN1

The choice to mix strain EbN1 with the enrichment culture UFZ-1 was based on the assumption that strain EbN1 would grow under denitrifying conditions exclusively on toluene, whereas UFZ-1 would grow under the same conditions exclusively on gluconate. As expected, no [$^{13}\text{C}_7$]-toluene-derived carbon was incorporated into proteins not related to strain EbN1. In contrast, ^{13}C incorporation into proteins from strain EbN1 varied between 78% and 83%. This is in good accordance with the expected results and is only slightly lower than the value from the pure culture grown only on ^{13}C (88–94%). As a reason for the slightly decreased incorporation rate, the usage of gluconate can be excluded because strain EbN1 lacks the genes for the consumption of gluconate (Rabus *et al.*, 2005) and therefore cannot grow on gluconate (Figure 1c). The culture UFZ-1 might disseminate metabolites of gluconate oxidation into the medium that can be assimilated by strain EbN1, for example acetate or pyruvate, thus lowering the actual level of ^{13}C -incorporation in proteins of EbN1. Also, the opposite carbon flow—dissemination of metabolites from strain EbN1 to the UFZ-1 culture—is principally possible but was actually not occurring within the detection limit of our model experiment, as no labelled proteins were found that were not related to strain EbN1. Despite the fact that strain EbN1 incorporated slightly lower amounts of ^{13}C into proteins in the mixed culture experiment compared with the pure culture experiment, estimating the amount of incorporated ^{13}C in the mixed culture experiment allowed us to sensitively prove specific toluene metabolization by strain EbN1 under denitrifying conditions. Therefore, by the method of protein-SIP, it is possible to link structural relationships with functional relationships within mixed bacterial cultures. We calculated the amount of incorporation required for a reliable detection of isotope labelling. A clear difference in the isotopic envelope of the peptides can be detected by a mass difference of 1 AMU or in respect to the size of the peptide in the incorporation of 1–2 atom % ^{13}C . This is significantly more sensitive than DNA-SIP that relies on the separation of heavy and light DNA by density-gradient centrifugation (Dumont and Murrell, 2005). The current limits of physical separation of labelled nucleic acids in density gradients result in a relatively low resolution of this method and therefore require an incorporation of at least 30%.

Our metabolic labelling approach will supplement other already existing concepts in the field of environmental proteomics. Owing to the ever-increasing genomic information and improved MS

techniques allowing shotgun analysis of metaproteomes, the focus of other studies was laid on analyzing microbial communities in terms of the identification of its members and of functionally involved proteins (Ram *et al.*, 2005; Wilmes and Bond, 2006a). The concept of protein-SIP allows monitoring of the metabolization of stable isotope-labelled substrates and transformation of these substrates into proteins of metabolically active members of a microbial community. Thus, the method permits to detect distinct physiological capacities within microbial communities, which are hardly or not detectable by focussing on protein expression patterns.

Determination of incorporation levels by comparing natural toluene with [$^{13}\text{C}_7$]-toluene fed cultures

In the case of metabolic labelling using single mass-based modified amino acids—for example, arginine or lysine—in cell cultures, incomplete labelling results in the presence of two peaks of tryptic peptide with a fixed mass difference. Algorithms exist that show how to use these changes in the database search for protein identification. However, the mass difference of potential stable isotope-labelled ^{13}C or ^{15}N -containing substrates (for example [$^{13}\text{C}_7$]-toluene, [$^{13}\text{C}_6$]-glucose, $^{15}\text{NH}_4^+$ or others) can vary widely, and techniques for the determination of incorporation levels are needed for the identification of the respective protein behind the peptide. This problem can be solved by comparing the labelled peptides with peptides from non-labelled controls, especially when a separation technique such as 2-DE is used. Our experimental set-up comprised separate growth experiments using non-labelled and ^{13}C -labelled substrate, separation of protein extracts by 2-DE and subsequent analysis of the protein extracts by MS, respectively (see scheme in Figure 5). After MS analysis, the data were compared and proteins could be identified on the basis of data of the non-labelled control experiment. Subsequently, the incorporation level in the ^{13}C experiment could be calculated, and the identification was validated based on the incorporation level (Figure 5). Another possibility is to make use of algorithms on the basis of the average, which allows conversion of any peptide mass into a theoretical sum formula and therefore into a theoretical isotopic distribution that can be used as an indicator of isotopic disturbance, pointing to incorporation of ^{13}C -atoms (Johnson and Muddiman, 2004). In principle, the incorporation can be calculated on the basis of the y_1 -ion of arginine and lysine, which would enable one to quantify incorporation levels by MS only.

To conclude, we have described a new method of metabolic labelling of proteins using ^{13}C -labelled substrate, which allows in principle to identify active species within bacterial consortia. Analyzing the increase of ^{13}C in proteins over time may be used

Figure 5 Scheme of protein-SIP analysis of mixed cultures with unknown metabolic networks but a known biochemical activity. Starting from a well-defined biochemical activity a substrate that is connected to the biochemical activity can be fed either on ^{12}C or ^{13}C -atoms. After growth the protein extracts are applied to 2-DE. Well-separated spots are excised, and the proteins were subsequently identified by mass spectrometry. This process yields the identity of the proteins and their nearest sequenced neighbour by cross-species identification, if the species is not sequenced by itself. In the final step, carbon fluxes within mixed cultures may be described and also predicted by modelling.

as a measure for enzyme stability and recycling of amino acids or to follow the specific induction of proteins after environmental changes.

Furthermore, we established a scheme for the determination of incorporation levels of ^{13}C by the subsequent analysis by 2-DE and mass spectrometry. The combination of these techniques might be helpful for the analysis of food chains, including the flow of ^{13}C or ^{15}N through natural bacterial communities based on the incorporation into proteins in general or in proteins of known functionality. Further future applications might involve the kinetic resolution of substrate usage and the description of subpopulations obtained by fluorescence-assisted cell sorting.

Acknowledgements

We thank Matthias Boll, University of Leipzig, for kindly providing us with strain EbN1. We also thank Stephanie Hinke, Michaela Risch, Yvonne Kullnick and Christine Schumann for excellent technical assistance. Furthermore, we thank Mathias Lukas for his kind support with data evaluation. This research was financially supported by the European Union (European Commission, Marie Curie Contract No. MTKD-CT 2006-042758).

References

- Barreiros L, Nogales B, Manaia CM, Ferreira ACS, Pieper DH, Reis MA *et al.* (2003). A novel pathway for mineralization of the thiocarbamate herbicide molinate by a defined bacterial mixed culture. *Environ Microbiol* **5**: 944–953.
- Benndorf D, Balcke GU, Harms H, von Bergen M. (2007). Functional metaproteome analysis of protein extracts from contaminated soil and groundwater. *ISMEJ* **1**: 224–234.
- Benndorf D, Müller A, Bock K, Manuwald O, Herbarth O, von Bergen M. (2008). Identification of spore allergens from the indoor mould *Aspergillus versicolor*. *Allergy* **63**: 454–460.
- Biegert T, Fuchs G, Heider F. (1996). Evidence that anaerobic oxidation of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by formation of benzylsuccinate from toluene and fumarate. *Eur J Biochem* **238**: 661–668.
- Boll M, Albracht SSP, Fuchs G. (1997). Benzoyl-CoA reductase (dearomatizing), a key enzyme of anaerobic aromatic metabolism—A study of adenosinetriphosphatase activity, ATP stoichiometry of the reaction and EPR properties of the enzyme. *Eur J Biochem* **244**: 840–851.
- Boschker HTS, Nold SC, Wellsbury P, Bos D, de Graaf W, Pel R *et al.* (1998). Direct linking of microbial populations to specific biogeochemical processes by C-13-labelling of biomarkers. *Nature* **392**: 801–805.
- Bradford MM. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254.
- Bull ID, Parekh NR, Hall GH, Ineson P, Evershed RP. (2000). Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature* **405**: 175–178.
- Cargile BJ, Bundy JL, Grunden AM, Stephenson JL. (2004). Synthesis/degradation ratio mass spectrometry for measuring relative dynamic protein turnover. *Anal Chem* **76**: 86–97.
- Drzyzga O, EL Mamouni R, Agathos SN, Gottschal JC. (2002). Dehalogenation of chlorinated ethenes and immobilization of nickel in anaerobic sediment columns under sulfidogenic conditions. *Environ Sci Technol* **36**: 2630–2635.
- Dumont MG, Murrell JC. (2005). Stable isotope probing—linking microbial identity to function. *Nat Rev Microbiol* **3**: 499–504.
- Fischer A, Herklotz I, Herrmann S, Thullner M, Weelink SAB, Stams AJM *et al.* (2008). Combined carbon and hydrogen isotope fractionation investigations for elucidating benzene biodegradation pathways. *Environ Sci Technol*; doi:10.1021/es702468f.
- Friedrich MW. (2006). Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. *Curr Opin Biotechnol* **17**: 59–66.
- Georgieva D, Risch M, Kardas A, Buck F, von Bergen M, Betzel C. (2008). Comparative analysis of the venom proteomes of *Vipera ammodytes ammodytes* and *Vipera ammodytes meridionalis*. *J Proteome Res* **7**: 866–886.
- Geyer R, Peacock AD, Miltner A, Richnow HH, White DC, Sublette KL *et al.* (2005). *In situ* assessment of biodegradation potential using biotrap amended with C-13-labeled benzene or toluene. *Environ Sci Technol* **39**: 4983–4989.
- Hanson JR, Macalady JL, Harris D, Scow KM. (1999). Linking toluene degradation with specific microbial populations in soil. *Appl Environ Microbiol* **65**: 5403–5408.
- Hehemann JH, Redecke L, Murugaiyan J, von Bergen M, Betzel C, Saborowski R. (2008). Autoproteolytic stability of a trypsin from the marine crab *Cancer pagurus*. *Biochem Biophys Res Commun* **370**: 566–571.
- Hugenholtz P, Goebel BM, Pace NR. (1998). Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J Bacteriol* **180**: 4765–4774.
- Hutchens E, Radajewski S, Dumont MG, McDonald IR, Murrell JC. (2004). Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. *Environ Microbiol* **6**: 111–120.
- Jimenez L, Breen A, Thomas N, Federle TW, Sayler GS. (1991). Mineralization of linear alkylbenzene sulfonate by a 4-member aerobic bacterial consortium. *Appl Environ Microbiol* **57**: 1566–1569.
- Johnson KL, Muddiman DC. (2004). A method for calculating O-16/O-18 peptide ion ratios for the relative quantification of proteomes. *J Am Soc Mass Spectrom* **15**: 437–445.
- Keller M, Zengler K. (2004). Tapping into microbial diversity. *Nat Rev Microbiol* **2**: 141–150.
- Koch R. (1881). Zur Untersuchung von pathogenen Organismen. *Mittheilungen aus dem kaiserlichen Gesundheitsamte* **1**: 1–48.
- Krüger M, Wolters H, Gehre M, Richnow HH. (2008). Tracing the slow growth of anaerobic methane oxidising communities by N-15 labelling techniques. *Fems Microbiol Ecol* **63**: 401–411.
- Lin JL, Radajewski S, Eshinimaev BT, Trotsenko YA, McDonald IR, Murrell JC. (2004). Molecular diversity of methanotrophs in Transbaikalian soda lake sediments and identification of potentially active populations by stable isotope probing. *Environ Microbiol* **6**: 1049–1060.
- Lo I, Denef VJ, VerBerkmoes NC, Shah MB, Goltsman D, DiBartolo G *et al.* (2007). Strain-resolved community proteomics reveals recombining genomes of acidophilic bacteria. *Nature* **446**: 537–541.
- Manefield M, Whiteley AS, Griffiths RI, Bailey MJ. (2002). RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl Environ Microbiol* **68**: 5367–5373.
- Miltner A, Richnow HH, Kopinke FD, Kastner M. (2005). Incorporation of carbon originating from CO₂ into different compounds of soil microbial biomass and soil organic matter. *Isotopes Environ Health Stud* **41**: 135–140.
- Neuhoff V, Arold N, Taube D, Ehrhardt W. (1988). Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* **9**: 255–262.
- Nijenhuis I, Stelzer N, Kastner M, Richnow HH. (2007). Sensitive detection of anaerobic monochlorobenzene degradation using stable isotope tracers. *Environ Sci Technol* **41**: 3836–3842.
- Ong SE, Mann M. (2005). Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* **1**: 252–262.

- Pace NR. (1997). A molecular view of microbial diversity and the biosphere. *Science* **276**: 734–740.
- Pelz O, Chatzinotas A, Andersen N, Bernasconi SM, Hesse C, Abraham WR *et al.* (2001a). Use of isotopic and molecular techniques to link toluene degradation in denitrifying aquifer microcosms to specific microbial populations. *Arch Microbiol* **175**: 270–281.
- Pelz O, Chatzinotas A, Zarda-Hess A, Abraham WR, Zeyer J. (2001b). Tracing toluene-assimilating sulfate-reducing bacteria using C-13-incorporation in fatty acids and whole-cell hybridization. *Fems Microbiol Ecol* **38**: 123–131.
- Pelz O, Tesar M, Wittich RM, Moore ER, Timmis KN, Abraham WR. (1999). Towards elucidation of microbial community metabolic pathways: unravelling the network of carbon sharing in a pollutant-degrading consortium by immunocapture and isotopic ratio mass spectrometry. *Environ Microbiol* **1**: 167–174.
- Pfennig N, Eimhjell Ke, Jensen SL. (1965). A new isolate of *Rhodospirillum Fulvum* group and its photosynthetic pigments. *Archiv Fur Mikrobiologie* **51**: 258.
- Rabus R. (2005). Functional genomics of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Appl Microbiol Biotechnol* **68**: 580–587.
- Rabus R, Kube M, Heider J, Beck A, Heitmann K, Widdel F *et al.* (2005). The genome sequence of an anaerobic aromatic-degrading denitrifying bacterium, strain EbN1. *Arch Microbiol* **183**: 27–36.
- Rabus R, Widdel F. (1995). Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. *Arch Microbiol* **163**: 96–103.
- Ram RJ, VerBerkmoes NC, Thelen MP, Tyson GW, Baker BJ, Blake RC *et al.* (2005). Community proteomics of a natural microbial biofilm. *Science* **308**: 1915–1920.
- Rappe MS, Giovannoni SJ. (2003). The uncultured microbial majority. *Annu Rev Microbiol* **57**: 369–394.
- Richnow HH, Vieth A, Kaestner M, Gehre M, Meckenstock RU. (2002). Isotope fractionation of toluene: a perspective to characterise microbial *in situ* degradation. *Scientific World Journal* **2**: 1227–1234.
- Santos PM, Roma V, Benndorf D, von Bergen M, Harms H, Sa-Correia I. (2007). Mechanistic insights into the global response to phenol in the phenol-biodegrading strain *Pseudomonas* sp. M1 revealed. *Omics* **11**: 233–251.
- Schagger H, Aquila H, von Jagow G. (1988). Coomassie blue sodium dodecyl-sulfate polyacrylamide-gel electrophoresis for direct visualization of polypeptides during electrophoresis. *Anal Biochem* **173**: 201–205.
- Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O *et al.* (1996). Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proc Natl Acad Sci USA* **93**: 14440–14445.
- Snijders APL, de Vos MGJ, de Koning B, Wright PC. (2005a). A fast method for quantitative proteomics based on a combination between two-dimensional electrophoresis and N-15-metabolic labelling. *Electrophoresis* **26**: 3191–3199.
- Snijders APL, de Vos MGJ, Wright PC. (2005b). Novel approach for peptide quantitation and sequencing based on N-15 and C-13 metabolic labeling. *J Proteome Res* **4**: 578–585.
- Snijders APL, Walther J, Peter S, Kinnman I, de Vos MGJ, van de Werken HJG *et al.* (2006). Reconstruction of central carbon metabolism in *Sulfolobus solfataricus* using a two-dimensional gel electrophoresis map, stable isotope labelling and DNA microarray analysis. *Proteomics* **6**: 1518–1529.
- Webster G, Watt LC, Rinna J, Fry JC, Evershed RP, Parkes RJ *et al.* (2006). A comparison of stable-isotope probing of DNA and phospholipid fatty acids to study prokaryotic functional diversity in sulfate-reducing marine sediment enrichment slurries. *Environ Microbiol* **8**: 1575–1589.
- Whiteley AS, Manefield M, Lueders T. (2006). Unlocking the ‘microbial black box’ using RNA-based stable isotope probing technologies. *Curr Opin Biotechnol* **17**: 67–71.
- Widdel F, Rabus R. (2001). Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotechnol* **12**: 259–276.
- Wilmes P, Bond PL. (2006a). Metaproteomics: studying functional gene expression in microbial ecosystems. *Trends Microbiol* **14**: 92–97.
- Wilmes P, Bond PL. (2006b). Towards exposure of elusive metabolic mixed-culture processes: the application of metaproteomic analyses to activated sludge. *Water Sci Technol* **54**: 217–226.
- Zengler K, Richnow HH, Rossello-Mora R, Michaelis W, Widdel F. (1999). Methane formation from long-chain alkanes by anaerobic microorganisms. *Nature* **401**: 266–269.

Supplementary Information accompanies the paper on The ISME Journal website (<http://www.nature.com/ismej>)