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A comparison of stable-isotope probing of DNA and phospholipid fatty acids to study prokaryotic functional diversity in sulfate-reducing marine sediment enrichment slurries

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Summary

Marine sediment slurries enriched for anaerobic, sulfate-reducing prokaryotic communities utilizing glucose and acetate were used to provide the first comparison between stable-isotope probing (SIP) of phospholipid fatty acids (PLFA) and DNA (16S rRNA and *dsrA* genes) biomarkers. Different ^{13}C-labelled substrates (glucose, acetate and pyruvate) at low concentrations (100 μM) were used over a 7-day incubation to follow and identify carbon flow into different members of the community. Limited changes in total PLFA and bacterial 16S rRNA gene DGGE profiles over 7 days suggested the presence of a stable bacterial community. A broad range of PLFA were rapidly labelled (within 12 h) in the ^{13}C-glucose slurry but this changed with time, suggesting the presence of an active glucose-utilizing population and later development of another population able to utilize glucose metabolites. The identity of the major glucose-utilizers was unclear as ^{13}C-enriched PLFA were common (16:0, 16:1, 18:1ω7, highest incorporation) and there was little difference between ^{12}C- and ^{13}C-DNA 16S rRNA gene denaturing gradient gel electrophoresis (DGGE) profiles. Seemingly glucose, a readily utilizable substrate, resulted in widespread incorporation consistent with the higher extent of ^{13}C-incorporation (~10 times) into PLFA compared with ^{13}C-acetate or ^{13}C-pyruvate. ^{13}C-PLFA in the ^{13}C-acetate and ^{13}C-pyruvate slurries were similar to each other and to those that developed in the ^{13}C-glucose slurry after 4 days. These were more diagnostic, with branched odd-chain fatty acids (*i*15:0, *a*15:0 and 15:1ω6) possibly indicating the presence of *Desulfococcus* or *Desulfosarcina* sulfate-reducing bacteria (SRB) and sequences related to these SRB were in the ^{13}C-acetate-DNA *dsrA* gene library. The ^{13}C-acetate-DNA 16S rRNA gene library also contained sequences closely related to SRB, but these were the acetate-utilizing *Desulfobacter* sp., as well as a broad range of uncultured *Bacteria.* In contrast, analysis of DGGE bands from ^{13}C-DNA demonstrated that the candidate division JS1 and *Firmicutes* were actively assimilating ^{13}C-acetate. Denaturing gradient gel electrophoresis also confirmed the presence of JS1 in the ^{13}C-DNA from the ^{13}C-glucose slurry. These results demonstrate that JS1, originally found in deep subsurface sediments, is more widely distributed in marine sediments and provides the first indication of its metabolism; incorporation of acetate and glucose (or glucose metabolites) under anaerobic, sulfate-reducing conditions. Here we demonstrate that PLFA- and DNA-SIP can be used together in a sedimentary system, with low concentrations of ^{13}C-substrate and overlapping incubation times (up to 7 days) to provide complementary, although not identical, information on carbon flow and the identity of active members of an anaerobic prokaryotic community.

Introduction

Sulfate reduction plays a major role in the global sulfur cycle, and may be regarded as one of the oldest metabolic processes on Earth (Castresana and Moreira, 1999; Shen *et al.*, 2001). Sulfate-reducing prokaryotes (SRP) constitute a large physiologically and phylogenetically diverse group of anaerobes utilizing a wide range of organic substrates (Rabus *et al.*, 2000). They are ubiquitous and

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important in almost all anoxic environments (Rabus *et al*., 2000) especially marine sediments (Ravenschlag *et al*., 2000).

Culture-independent approaches based on 16S rRNA gene sequencing and phospholipid fatty acid (PLFA) profiles have been widely used to characterize sulfate-reducing bacteria (SRB) communities, including those in marine sediments (Parkes *et al*., 1993; Ravenschlag *et al*., 2000; Purdy *et al*., 2001). However, these methods do not provide an unambiguous link to the physiology or metabolic capabilities of a bacterium, particularly in phylogenetic lineages without cultured representatives. Functional genes encoding enzymes catalysing key activities have been successfully used to detect biogeochemically important prokaryotes in complex habitats. For example, SRP have been identified by analysis of dissimilatory sulfite reductase (*dsrAB*) gene sequences (Wagner *et al*., 1998) in estuarine and marine sediments (Joulian *et al*., 2001; Thomsen *et al*., 2001; Nercessian *et al*., 2005). However, the presence of functional genes within an environmental sample does not necessarily mean that the organisms from which they are derived are active in that system.

Recently, the technique of stable-isotope probing (SIP) has been used to link functional activity to microbial community structure by adding ^{13}C-labelled substrates to environmental samples and identifying specifically labelled biomarkers (Boschker *et al*., 1998; Radajewski *et al*., 2000; Dumont and Murrell, 2005). Using this approach, Boschker and colleagues (1998) characterized the microbial community responsible for acetate oxidation in coastal sediments after incorporation of ^{13}C-acetate, and concluded from ^{13}C-enriched PLFA signature profiles that *Desulfotomaculum acetoxidans* was the dominant acetate oxidizer. Radajewski and colleagues (2000) added ^{13}C-labelled methanol and CH_4 to soil microcosms and identified specific active populations of methylotrophic bacteria from their labelled DNA, while Manefield and colleagues (2002) detected active microbial populations in an aerobic phenol-degrading bioreactor from ^{13}C-labelled RNA molecules. Increasingly, other important physiological groups of prokaryotes are also being investigated using similar techniques, including the methanogenic *Archaea* (Lu *et al*., 2005), sulfur oxidizing (Knief *et al*., 2003), nitrifying (Whitby *et al*., 2001) and denitrifying bacteria (Gallagher *et al*., 2005).

To date, PLFA, DNA and RNA biomarkers have only been used separately for SIP to investigate active species in microbial populations (Boschker *et al*., 1998; Manefield *et al*., 2002; Radajewski *et al*., 2002; Treonis *et al*., 2004; Pombo *et al*., 2005), partly because different concentrations of ^{13}C-label and varying incubation times are needed for optimum PLFA and nucleic acid labelling (Boschker and Middelburg, 2002; Radajewski *et al*., 2003). As membrane lipids and 16S rRNA genes are both widely used as taxonomic biomarkers to identify bacteria, such as SRB, to the genus level (Kohring *et al*., 1994), it is important to assess the extent to which PLFA-SIP and DNA-SIP approaches result in identification of the same active organisms. Phospholipid fatty acid-based identification can be ambiguous particularly when applied to complex populations (Frostegard *et al*., 1993; Pombo *et al*., 2002), but offers a phenotypic index of identity and physiological information about a population, such as nutritional status and stress (Parkes, 1987). Analysis of rRNA genes and other genetic markers despite having biases associated with DNA extraction, polymerase chain reaction (PCR) and cloning seems to provide a more robust description of phylogenies (Dahlöff, 2002). Therefore, conducting SIP with both PLFA and DNA biomarkers may provide unique information about a prokaryotic community.

In this study we used SIP in combination for the first time with PLFA and DNA biomarkers to determine the active bacterial community structure within estuarine sediment sulfate-reducing slurry enrichments. Active bacterial populations were characterized using relatively short incubation times with low concentrations of different ^{13}C-labelled organic substrates.

Results

Bacterial activity in sediment slurries

All Tamar estuary sediment slurries were pre-enriched with both glucose and acetate prior to ^{13}C-substrate addition under anaerobic conditions to stimulate a simplified sulfate-reducing community and ease interpretation. Between 2 and 6 days of pre-enrichment, all sediment slurries had approximately a 10-fold increase in bacterial numbers from 7.7×10^7 cells per gram of dry weight sediment, after which the cell numbers remained relatively stable. Sulfate reduction became intense by 7 days with a marked decrease in sulfate concentrations (6.6–7.3 mM SO_4^{2-} per day) and a steady increase in dissolved sulfide (~2.6 mM S^{2-} per day). After further 5 days of sulfate reduction (sulfate concentrations < 2 mM), labelled ^{13}C-substrates (^{13}C-glucose, ^{13}C-acetate and ^{13}C-pyruvate) were added to a final concentration of 100 μM and incubated for further 7 days during which time all sulfate was removed.

Total PLFA profiles of sediment slurries

Throughout the incubations with ^{13}C-labelled substrates, PLFA profiles of the sediment slurries were monitored and there were changes in the distribution of PLFAs with 16:0, 16:1, 18:0 and 18:1ω7 being major components in all slurries (Fig. 1A and B). This profile is similar to patterns observed previously for the Tamar estuary (Boschker

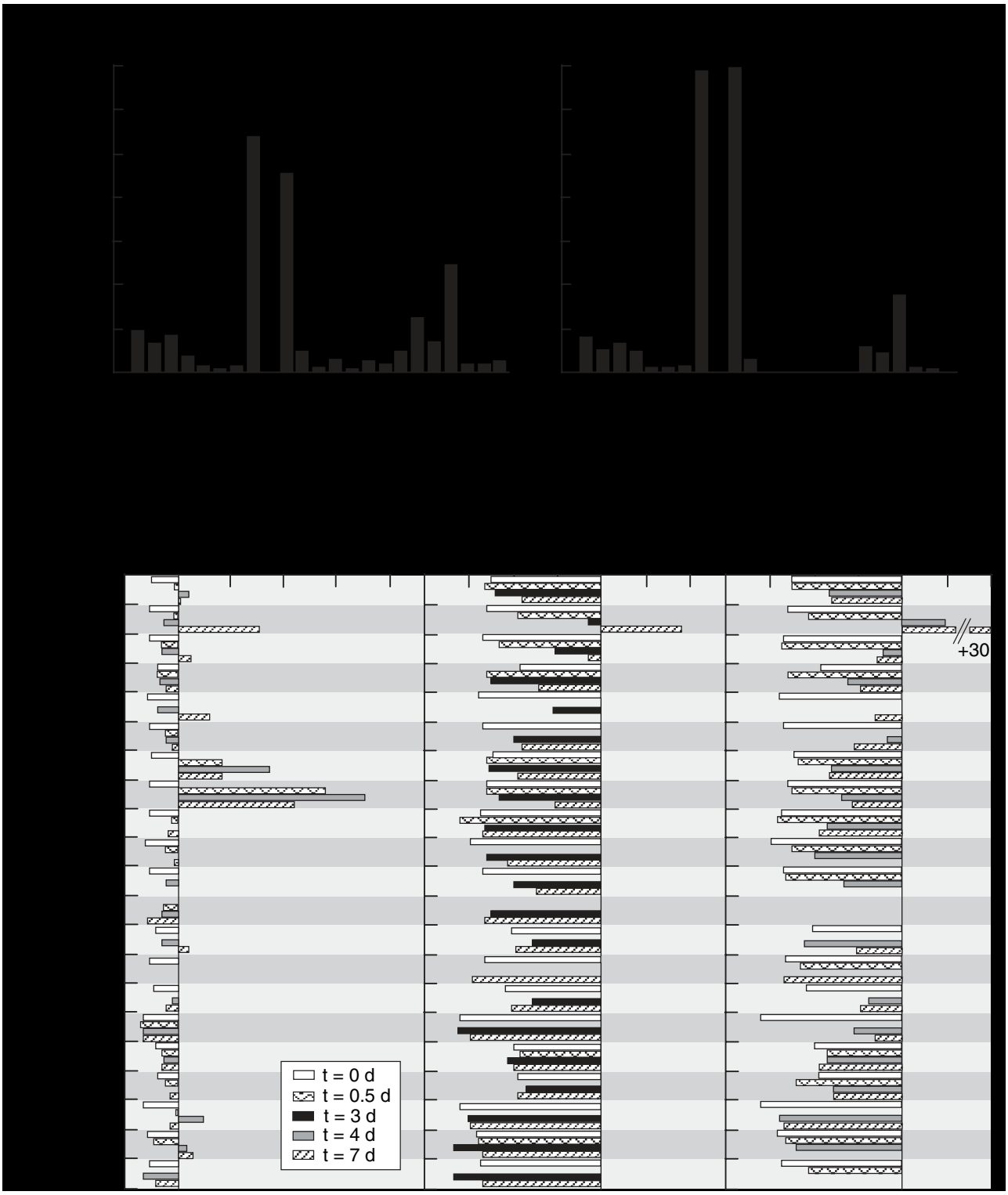

Fig. 1. Phospholipid fatty acid abundance as per cent of total PLFA in sediment slurries 4 days after ^{13}C substrate addition: (A) ^{13}C-glucose sediment slurry, (B) ^{13}C-acetate sediment slurry. Values of $\delta^{13}C$ measured in PLFA extracted at different time points from sediment slurries prior to and after addition of (C) ^{13}C-glucose, (D) ^{13}C-acetate and (E) ^{13}C-pyruvate.

et al., 1998) and other marine sediments (Guckert *et al*., 1985; Parkes *et al*., 1993).

Isotopic enrichment of PLFA

The average $\delta^{13}C$ value of PLFA from the pre-^{13}C-labelled samples was –25.6 ± 4‰ (Fig. 1). In all experiments after 12 h of incubation there was detectable ^{13}C enrichment in PLFA molecules (Fig. 1C–E). Although, greater (~10-fold) incorporation of $\delta^{13}C$ occurred with ^{13}C-glucose compared with other labelled substrates. Samples from the slurry incubated with ^{13}C-glucose at 12 h and 4 days showed that the majority of the ^{13}C-label (82–87%) was in even-chain fatty acids with 56–58% in unsaturated PLFA, 15–18% in branched chain fatty acids and 24–27% in saturated fatty acids. The highest $\delta^{13}C$ values were in the 16:1ω7 (+140‰ and +177‰), 16:0 (+43‰ and +87‰) and 18:1ω7c (–0.3‰ and +25‰) PLFAs. However, after a 7-day incubation with ^{13}C-glucose the PLFA *i*15:0 (+78‰) and *a*15:0 (+13‰) became noticeably more enriched and contained 17.5% and 6% of the ^{13}C-label respectively (Fig. 1C). This coincided with a decrease in the ^{13}C content of previously highly labelled PLFAs.

Enriched PLFA profiles from slurries incubated with ^{13}C-acetate or ^{13}C-pyruvate were almost identical throughout the 7-day incubation period with the extent of enrichment generally increasing. By 3–4 days incubation, 48–50% of the ^{13}C-label was in the branched chain fatty acids with the majority being in odd-chain fatty acids (Fig. 1D and E). For example, the major $\delta^{13}C$ PLFA compounds for the ^{13}C-acetate sediment slurry after 3 days incubation were *i*15:0 (–2.8‰), *a*15:0 (–10.5‰) and 15:1ω6 (–11.1‰) with a small amount of $\delta^{13}C$ in 17:0, 17:1ω7 and br17:0 (Fig. 1D). A similar ^{13}C-enriched PLFA profile occurred in the ^{13}C-pyruvate slurry with the exception that after 7 days slightly more $\delta^{13}C$ was present in 17:1ω6 and 17:1ω7 than in the ^{13}C-acetate enrichment (Fig. 1E).

Denaturing gradient gel electrophoresis (DGGE) profiles of sediment slurries

Changes in bacterial populations of the sulfate-reducing sediment slurries were also monitored throughout the incubations with ^{13}C-substrates using PCR-DGGE analysis of 16S rRNA genes (data not shown). During 7 days incubation no change in the DGGE profile was observed for the ^{13}C-glucose sediment slurry compared with that prior to ^{13}C-glucose addition (0 h). However, after only 12 h incubation with ^{13}C-acetate or ^{13}C-pyruvate there was some evidence of a change in the sediment population compared with time zero. New DGGE bands appeared, suggesting that growth of these organisms was stimulated by the utilization of either ^{13}C-acetate or ^{13}C-pyruvate (two and three new bands respectively). The bacterial populations stimulated in these enrichments were stable for the remainder of the 7-day incubation. It is noteworthy that a DGGE band corresponding to the uncultured bacterial candidate division JS1, distantly related to the uncultured candidate division OP9 (Webster *et al*., 2004) was present in all slurries at all time points.

Optimization of CsCl density gradient ultracentrifugation for separation of ^{12}C- and ^{13}C-labelled DNA

Separation of ^{13}C-DNA from the unlabelled ^{12}C-DNA is a critical step in DNA-SIP. Therefore, it was necessary to demonstrate effective separation of labelled and unlabelled DNA and to optimize the methodology of CsCl-ethidium bromide density gradient ultracentrifugation. Optimization was also necessary as only limited concentrations of labelled DNA would be recovered from sediments enriched with low ^{13}C-substrate concentrations. Separation of DNA mixtures containing *Pseudomonas putida* ^{13}C-DNA and *Mycobacterium smegmatis* ^{12}C-DNA was readily achieved using the conditions described. Both fractions were removed from the CsCl gradient and PCR-amplified with bacterial 16S rRNA gene primers and analysed by DGGE (Fig. 2). The results show that there was no contamination of the ^{13}C-DNA fraction with DNA derived from the ^{12}C-DNA of *M. smegmatis* and vice versa, although there was both ^{12}C- and ^{13}C-DNA in the

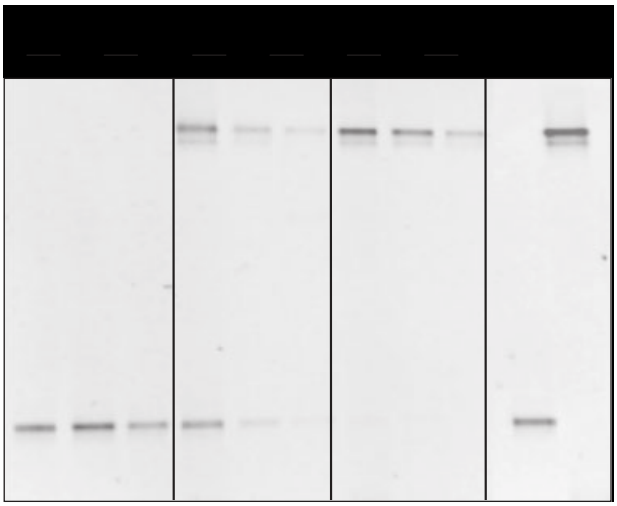

Fig. 2. Polymerase chain reaction-DGGE analysis of 16S rRNA genes amplified from three fractions removed from CsCl/ethidium bromide density gradient separation of ^{12}C- and ^{13}C-DNA extracted from pure cultures of *Mycobacterium smegmatis* and *Pseudomonas putida* respectively. Lane numbers represent the sampling points from where DNA fractions were removed. Lanes marked 1, DNA fraction 1 (^{12}C-DNA band); Lanes marked 2, DNA fraction 2 (between ^{12}C- and ^{13}C-DNA bands); Lanes marked 3, DNA fraction 3 (^{13}C-DNA band); Lane *Ms*, *M. smegmatis*; Lane *Pp*, *P. putida*. Denaturing gradient gel electrophoresis analyses of fractions are shown in triplicate from PCRs on DNA template diluted 10^0, 10^{-1}, 10^{-2} prior to amplification (left to right).

zone between the two fractions. Lueders and colleagues (2004) quantitatively analysed DNA fractions from CsCl gradients by real-time PCR and obtained similar findings when fractions were sampled carefully.

Separation of ^{13}C-labelled SRB and sediment DNA

DNA extracted from a pure culture of the acetoclastic *Desulfobacter* sp. grown on ^{12}C- and ^{13}C-labelled acetate was fractionated by CsCl-density gradient ultracentrifugation, confirming that acetate-utilizing SRB are amenable to the DNA-SIP approach (Fig. 3A and C; tube 1). DNA was only extracted from the ^{13}C-substrate sediment slurries after 7 days incubation; it was considered at this time point that there might be sufficient incorporation of ^{13}C into sulfate-reducing prokaryotic DNA to be separated from the ^{12}C-DNA. Subsequently, it has been shown that sufficient ^{13}C can be incorporated into DNA of benzoate-utilizing denitrifying bacteria after 1 h incubation and identified by SIP (Gallagher *et al*., 2005). DNA extracted from the ^{13}C-labelled sediment slurries (glucose and acetate only) and centrifuged in a CsCl-ethidium bromide density gradient was visible as a faint diffuse ^{12}C band (Fig. 3A and C). This suggested that the sediment DNA was comprised of unlabelled, partially labelled and fully ^{13}C-labelled DNA, similar to the intermediately labelled DNA observed in other SIP experiments (Morris *et al*., 2002; Hutchens *et al*., 2004). Despite the apparent absence of a distinct ^{13}C-DNA band, fractions were collected from the CsCl gradients guided by a 'marker' density gradient, containing *Desulfobacter* sp. ^{12}C- and ^{13}C-DNA (Fig. 3A and C).

Molecular analysis of ^{13}C-DNA fractions

Bacterial 16S rRNA genes from the ^{13}C- and ^{12}C-DNA fractions from the ^{13}C-labelled glucose and acetate slurries were amplified by PCR and analysed by DGGE. Analysis of the ^{13}C-glucose slurry (Fig. 3B) showed that both fractions had identical profiles suggesting that all members of the dominant bacterial community were

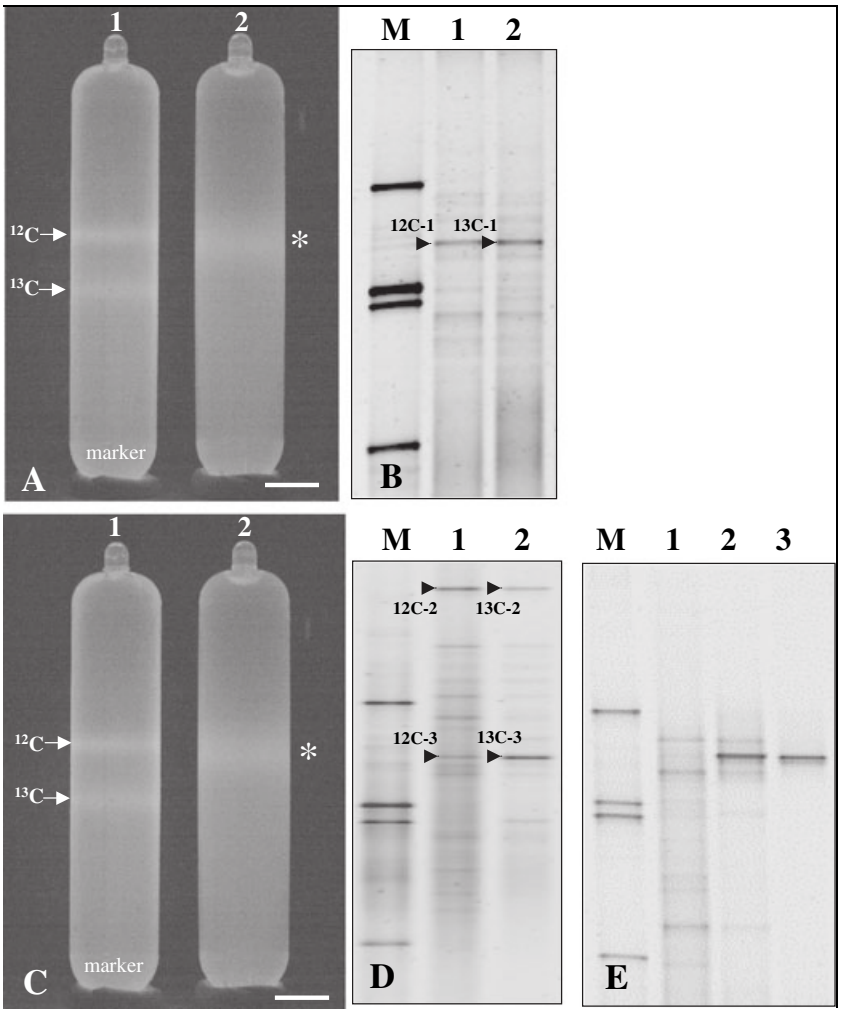

Fig. 3. Sediment DNA in CsCl/ethidium bromide density gradients after equilibrium centrifugation, and subsequent DGGE analysis of ^{12}C- and ^{13}C-DNA fractions.
A. DNA extracted (tube 2; asterisk highlights DNA band) from the Tamar estuary sediment slurry incubated with ^{13}C-glucose for 7 days alongside a tube containing DNA extracted from the SRB, *Desulfobacter* sp. DSM 2035 (grown on either ^{12}C- or ^{13}C-acetate as the sole carbon source) marker tube (tube 1) as a visual guide.
B. Denaturing gradient gel electrophoresis analysis of 16S rRNA genes amplified by PCR primers 357F/518R from the ^{12}C- and ^{13}C-DNA from the ^{13}C-glucose sediment slurry (tube 2 in A).
C. DNA extracted (tube 2; asterisk highlights DNA band) from the Tamar estuary sediment slurry incubated with ^{13}C-acetate for 7 days alongside the *Desulfobacter* sp. DNA marker tube (tube 1) as a visual guide.
D. Denaturing gradient gel electrophoresis analysis of 16S rRNA genes amplified by PCR primers 357F/518R from the ^{12}C- and ^{13}C-DNA from the ^{13}C-acetate sediment slurry (tube 2 in C).
E. Denaturing gradient gel electrophoresis analysis of 16S rRNA genes amplified by nested PCR with JS1 primers 63F/665R and 357F/518R from the ^{12}C- and ^{13}C-DNA from the ^{13}C-acetate sediment slurry (tube 2 in C).
Lanes marked M, DGGE marker (Webster *et al*., 2003); 1, ^{12}C-DNA fraction; 2, ^{13}C-DNA fraction; 3, JS1 clone NankB-7 (Newberry *et al*., 2004). Labelled DGGE bands represent bands that were excised and sequenced (see Table 1). Bar = 1 cm.

Table 1. Closest 16S rRNA gene sequence matches to excised DGGE bands using the BLASTN search tool.

DGGE band identifier	Sulfate-reducing sediment slurry (DNA fraction)	Nearest two matches by BLASTN search (accession no.)	Sequence similarity (%)	Alignment length (bp)	Phylogenetic affiliation	Isolation environment of nearest matches
12C-1	^{13}C-Glucose (^{12}C-DNA)	Uncultured bacterium clone ChCM-5 (AJ605563)	97	157	JS1 candidate division	Marine sediment, Chile Continental Margin
		Uncultured bacterium clone MB-B2-103 (AY093469)	96	157		Methane-hydrate bearing deep marine sediment, Nankai Forearc Basin
13C-1	^{13}C-Glucose (^{13}C-DNA)	Uncultured bacterium clone ChCM-5 (AJ605563)	100	168	JS1 candidate division	Marine sediment, Chile Continental Margin
		Uncultured bacterium clone MB-B2-103 (AY093469)	99	168		Methane-hydrate bearing deep marine sediment, Nankai Forearc Basin
12C-2	^{13}C-Acetate (^{12}C-DNA)	Uncultured bacterium clone PeM37 (AJ576396)	87	149	*Firmicutes*	*Pachnoda ephippiata* midgut
		Firmicutes sp. oral clone BX005 (AY005049)	90	115		Human subgingival plaque
12C-3	^{13}C-Acetate (^{12}C-DNA)	Uncultured bacterium clone ChCM-5 (AJ605563)	100	165	JS1 candidate division	Marine sediment, Chile Continental Margin
		Uncultured bacterium clone MB-B2-103 (AY093469)	99	165		Methane-hydrate bearing deep marine sediment, Nankai Forearc Basin
13C-2	^{13}C-Acetate (^{13}C-DNA)	Uncultured bacterium clone ZB13 (AY327215)	90	174	*Firmicutes*	Sulfide and sulfur-rich spring, Zodletone spring, Oklahoma
		Firmicutes sp. oral clone BX005 (AY005049)	91	112		Human subgingival plaque
13C-3	^{13}C-Acetate (^{13}C-DNA)	Uncultured bacterium clone ChCM-2 (AJ605563)	99	175	JS1 candidate division	Marine sediment, Chile Continental Margin
		Uncultured bacterium clone MB-B2-103 (AY093469)	98	175		Methane-hydrate bearing deep marine sediment, Nankai Forearc Basin

active and able to utilize glucose directly or indirectly via glucose degradation products. Sequencing the most intense DGGE band in the ^{12}C- and ^{13}C-DNA from the ^{13}C-glucose slurry (Table 1) revealed that this PCR product had a high sequence similarity (96–100%) to members of the bacterial candidate division JS1 found in deep marine sediments (Reed *et al*., 2002; Webster *et al*., 2004).

Figure 3D shows that the acetate-utilizing bacterial population in the ^{13}C-acetate sediment slurry was distinct from the total bacterial community structure (^{12}C-DNA fraction). The ^{13}C-acetate DGGE profile suggested that there was a subset of the bacterial population incorporating the ^{13}C-label, which produced two intensely stained DGGE bands against a background of other very faint bands. Sequencing of these prominent DGGE bands (Table 1) revealed that the bacteria able to utilize the ^{13}C-acetate under sulfate-reducing conditions belonged to two different phyla; JS1 and *Firmicutes*. The sequences obtained were most similar to uncultivated bacterial phylotypes and not to cultivated SRB. Denaturing gradient gel electrophoresis band 13C-2 showed 90% sequence similarity to clone ZB13 previously derived from a sulfur-rich spring (Elshahed *et al*., 2003), and DGGE band 13C-3 had a high similarity to JS1 sequences from the Chilean Continental Margin (Webster *et al*., 2004) and the Nankai Trough (Reed *et al*., 2002).

Analysis of a small bacterial 16S rRNA gene library (20 clones) from the ^{13}C-acetate slurry DNA fraction did not identify any JS1 sequences (Table 2), although four *Firmicutes*-like sequences were identified. Interestingly, some sequences were very closely related (97%) to known acetate-utilizing SRB *Desulfobacter postgatei* and *Desulfobacter latus* (Table 2). Other sequences included uncultured members of the *Gammaproteobacteria, Bacteroidetes, Actinobacteria, Alphaproteobacteria* and *Acidobacteria*.

Further investigation of the ^{13}C-acetate slurry using PCR primers targeted to JS1 16S rRNA genes confirmed the presence of active JS1 within this sediment. Analysis of 10 randomly selected clones from the ^{13}C-DNA fraction revealed that they were all closely related to each other (98.1–99.8%), and had a high similarity (> 97%) to phylotypes previously retrieved from sediment of the Chile Continental Margin (Webster *et al*., 2004), Sea of Okhotsk (Inagaki *et al*., 2003) and Nankai Trough (Reed *et al*., 2002; Newberry *et al*., 2004) suggesting they were from the same bacterial species. Further evidence indicating that JS1 was a member of the acetate-utilizing population in the sediment slurry was obtained by DGGE analysis of

Table 2. Closest 16S rRNA gene sequence matches to clone library sequences from ^{13}C-DNA extracted from the ^{13}C-acetate-enriched sulfate-reducing sediment slurry.

16S rRNA gene clone	Nearest matches by BLASTN search (accession no.)	Sequence similarity (%)	Phylogenetic affiliation
13C16S1-3	*Desulfobacter postgatei* (AF418180)	97	*Deltaproteobacteria*
13C16S1-4	Arctic pack ice clone ARKIA-79 (AF468290)	96	*Gammaproteobacteria*
13C16S1-7	*Moorella* sp. AIP 246.00 (AY766037)	88	*Firmicutes*
13C16S1-16, 13C16S1-52	Deep sediment clone JTB215 (AB015270)	96–97	*Firmicutes*
13C16S1-26, 13C16S-53	*Desulfobacter latus* (AJ441315)	97	*Deltaproteobacteria*
13C16S1-28	*Flavobacteria* clone SIMO-1405 (AY710845)	94	*Bacteroidetes*
13C16S1-30	Carbonate-rich sediment clone pIR3BC03 (AY354165)	96	*Actinobacteria*
13C16S1-31	Uncultured *Bacteroidetes* clone *Paralvinella palmiformis* A/C 22 (AJ441238)	92	*Bacteroidetes*
13C16S1-32, 13C16S1-55	Sulfur-oxidizing endosymbiont (AY129120)	94	*Gammaproteobacteria*
13C16S1-33	*Clostridium ganghwensis* (AY903294)	96	*Firmicutes*
13C16S1-35	Tidal flat sediment clone JH12_C101 (AY568933)	91	*Acidobacteria*
13C16S1-36	*Brevundimonas diminuta* (X87274)	99	*Alphaproteobacteria*
13C16S1-37	Sediment clone P21 (AY553949)	98	*Actinobacteria*
13C16S1-38, 13C16S1-51	Arctic Ocean sediment clone Sva1007 (AJ241019)	98	*Actinobacteria*
13C16S1-39	Bacteroidetes bacterium Ko710 (AF550591)	96	*Bacteroidetes*
13C16S1-45	Seagrass rhizosphere clone B2M54 (AF223298)	98	*Gammaproteobacteria*

JS1 PCR products derived from both the ^{12}C- and ^{13}C-DNA fractions (Fig. 3E). This revealed that JS1 16S rRNA gene sequences dominated the ^{13}C-DNA fraction, whereas the ^{12}C-DNA fraction DGGE profile was made up of a diverse mixture of 16S rRNA genes. The JS1 targeted PCR primers are also known to amplify other sequences outside the JS1 candidate division (Webster *et al*., 2004), and therefore the limited number of sequences amplified from ^{13}C-DNA by comparison with ^{12}C-DNA showed the former represented a much less diverse bacterial population.

Phylogenetic analysis of 22 partial *dsrA* sequences from the ^{13}C-acetate slurry ^{13}C-DNA fraction revealed a large diversity of *dsrA* genes (Fig. 4), which when subject to rarefaction analysis predicted that the majority of phylotypes had been sampled (data not shown). Most of the *dsrA* sequences (59%) from the ^{13}C-DNA fraction formed three distinct groups within *Desulfobacteraceae*: a *Desulfobacter*-like cluster (five sequences) related to *D. latus*, one sequence similar to *D. postgatei* and two clusters similar to environmental *dsrA* gene clones from marine sediments. A *Desulfosarcina*-like cluster with six ^{13}C-DNA-derived sequences similar to phylotypes from sediment of Kysing Fjord (Joulian *et al*., 2001) and another cluster (two sequences) within the uncultured group I *dsrA* sequences (Thomsen *et al*., 2001) from Kysing Fjord and anaerobic methane-oxidizing sediment from Aarhus Bay, Denmark. However, *Desulfococcus multivorans* also falls in this cluster (Fig. 4). Other *dsrA* genes from the ^{13}C-acetate-enriched sediment slurry DNA fraction included sequences that grouped with Guaymas Basin clone B01P021 (Dhillon *et al*., 2003) and associated with members of the *Syntrophobacteraceae*, which includes other known SRB. Lastly, some deeply branching, phylogentically distinct *dsrA* sequences were related to uncultured *dsrA* groups III and IV (Thomsen *et al*., 2001; Dhillon *et al*., 2003) from Kysing Fjord, Aarhus Bay and Guaymas Basin.

Phylogenetic analysis of *dsrA* genes from the ^{12}C-DNA fraction showed a similar level of diversity as in the ^{13}C-DNA *dsrA* gene library (Fig. 4); however, rarefaction analysis predicted that not all probable unique phylotypes had been found (data not shown) in the ^{12}C-DNA *dsrA* gene library. It is worth noting that two clones in the ^{12}C-DNA library were assigned to the family *Desulfobulbaceae*, which comprises SRB that carry out incomplete oxidation and are unable to utilize acetate for sulfate reduction.

Discussion

In this study we have shown, for the first time, that PLFA- and DNA-SIP can be used together to analyse active prokaryotic populations in a simplified sedimentary system, using low concentrations of ^{13}C-labelled substrates and overlapping incubation times. On the basis of total PLFA and PCR-DGGE profiles none of the labelled substrates resulted in major changes to the bacterial community in sediment slurries. The major PLFAs remained the same (Fig. 1A and B) and DGGE profiles following ^{13}C-substrate addition were the same (^{13}C-glucose) or similar (^{13}C-acetate and ^{13}C-pyruvate) to samples taken prior to addition. However, the ^{13}C-PLFA labelling patterns showed distinct changes in δ^{13}C incorporation with all ^{13}C-substrates.

In ^{13}C-glucose sediment slurries, ^{13}C-PLFA profiles changed distinctly with time, indicating altered patterns of carbon flow. Thus, in the 0.5–4 days period the bacterial population showed marked δ^{13}C incorporation into the main PLFA compounds (e.g. 16:1ω7c, 16:0 and 18:1ω7c) present in the sulfate-reducing sediment slurries prior to

Fig. 4. Phylogenetic tree showing the relationship of the *dsrA* sequences from the ^{12}C- and ^{13}C-DNA fractions extracted from the ^{13}C-acetate-enriched sulfate-reducing sediment slurry to pure culture sulfate-reducing bacteria and environmental clone sequences. The minimum evolution tree was derived by LogDet/Paralinear distances of variable sites (estimated value of proportion of invariable sites = 0.1809). The tree is one of 72 equally good trees differing only by minor re-arrangements of some branches and is based on 435 aligned nucleotides. Bootstrap support values over 50% (1000 replicates) are shown, NS denotes support below 50%. First value, bootstrap derived by LogDet/Paralinear distances of variable sites; second value, derived by maximum parsimony of translated amino acid sequence. Sequences of *Thermodesulfovibrio islandicus* (AF334599) and *Thermodesulfovibrio yellowstonii* (U58122) were used as outgroup. Uncultured *dsrA* groups I, III and IV are as described in studies by Thomsen and colleagues (2001) and Dhillon and colleagues (2003). (□) ^{12}C-DNA *dsrA* gene clones; (●) ^{13}C-DNA *dsrA* gene clones.

labelling (data not shown). However, after 7 days this changed to incorporation into *i*15:0, *a*15:0, 15:1ω6 and 17:0, with a decrease in the previously labelled PLFAs. This may be explained by a change in the bacterial population from one dominated by organisms utilizing glucose directly, to domination by organisms utilizing glucose fermentation or oxidation by-products (e.g. pyruvate, lactate, ethanol and acetate). The acetate-oxidizing *Dm. acetoxidans* has the same major PLFAs (Dowling *et al*., 1986) as were present in the 0.5–4 days period and has been suggested to be important in estuarine and aquifer sediments based on ^{13}C-acetate incorporation into PLFA (Boschker *et al*., 1998; Pombo *et al*., 2005). However, it seems unlikely that *Dm. acetoxidans* is dominant in the ^{13}C-glucose slurry as it does not utilize glucose as an electron donor for sulfate-reduction or fermentation (Widdel and Pfennig, 1977; 1981). Also, the most dominant PLFA in *Dm. acetoxidans* (Dowling *et al*., 1986) is 16:0, whereas the highest δ^{13}C PLFA in the ^{13}C-glucose slurry was 16:1ω7c. It is possible that the ^{13}C-labelled PLFA pattern is due to other uncultured or uncharacterized SRP. Recently, it has been shown that 16:1ω7c and 18:1ω7c are major PLFAs in some thermophilic *Desulfotomaculum* strains that grow on glucose (Goorissen *et al*., 2003) and in *Desulfobulbus mediterraneus*, a glucose-utilizing SRB (Sass *et al*., 2002). In addition, these PLFAs are common to a wide range of bacteria, including complex compound-degrading fermentative and acetogenic bacteria (James, 2001; Spring *et al*., 2003), many of which utilize glucose (Nealson, 1997; Chin *et al*., 1999). The odd-chain PLFAs observed in the ^{13}C-glucose sediment slurry after 7 days are less common than the even-chain fatty acids that they displaced and are present in some SRB. For example, *Ds. multivorans* contains high amounts of all four of the ^{13}C-PLFAs (5% *i*15:0, 30% *a*15:0, 5% 15:1ω6 and 1% 17:0; Rütters, 2001), and also has a broad substrate range, including lactate, ethanol and fatty acids (C3-C16; Rabus *et al*., 2000). The observed relative decrease in even-chain δ^{13}C PLFAs might also indicate recycling of ^{13}C from the degradation of a previously labelled ^{13}C-glucose-utilizing organism's biopolymers or from a combination of both carbon recycling and carbon flow from ^{13}C-glucose.

In contrast to the ^{13}C-PLFA profile changes, the DGGE profile of the dominant bacterial 16S rRNA genes in the ^{13}C-DNA fraction from the ^{13}C-glucose sediment slurry was identical to that of the ^{12}C-DNA fraction (Fig. 3B), suggesting that the major bacterial groups were all actively utilizing glucose or its metabolites. Interestingly, a prominent band in both the ^{12}C- and ^{13}C-DNA fractions was assigned to the new candidate division JS1 (Webster *et al*., 2004). This uncultured group of *Bacteria* has only been shown to be present in anoxic environments, including deep subseafloor (Reed *et al*., 2002; Inagaki *et al*., 2003; Newberry *et al*., 2004; Parkes *et al*., 2005), brackish and coastal sediments (Webster *et al*., 2004), which is consistent with its presence in our estuarine sediment slurries.

Because acetate is the principal substrate for sulfate reduction and a key intermediate in the degradation of organic matter in marine sediments (Parkes *et al*., 1989), detailed molecular analysis of the ^{13}C-acetate slurry was conducted. In this and the ^{13}C pyruvate slurry, odd-chain and branched fatty acids (*i*15:0, *a*15:0, 15:1ω6) increasingly dominated the labelled PLFA profiles throughout the 7 days (Fig. 1D and E), suggesting active bacterial populations similar to those at 7 days with ^{13}C-glucose. Previous ^{13}C-acetate SIP in Tamar estuary and German Baltic sea sediments demonstrated labelling of 16:1ω7, 16:1ω5, 16:0 and 18:1ω7, characteristic of *Dm. acetoxidans* (Boschker *et al*., 1998; Boschker *et al*., 2001). Other workers have shown that the acetoclastic SRB *Desulfobacter* dominate estuarine sediments (Purdy *et al*., 2001), acetate-amended slurries (Parkes *et al*., 1993) and are commonly isolated on acetate from marine sediments (Widdel, 1987). However, *Desulfobacter* species are dominated by even-chain fatty acids, particularly 16:0, which can represent 30% of the total PLFA (Kohring *et al*., 1994), and this was not evident in the ^{13}C-acetate slurry ^{13}C-PLFA profile. It should be noted that *Desulfobacter* species grown on acetate in the presence of other volatile fatty acids (VFA), such as propionate, can change the PLFA profile to favour odd-numbered and branched fatty acids including *i*15:0, *a*15:0 and 15:1ω6, by incorporation as chain initiators (Dowling *et al*., 1986). Low concentrations of propionate and other VFA were present in the slurries (data not shown), presumably from breakdown of glucose in the initial pre-enrichment, and this may have resulted in limited formation of odd, branched chain PLFAs. However, the ^{13}C-acetate-enriched PLFA profile probably indicates

the presence of other active SRB populations (e.g. *Ds. multivorans*) or other prokaryotes similar to those in the later stages of the ^{13}C-glucose slurry.

The 16S rRNA and *dsrA* gene libraries from the ^{13}C-DNA fraction supported the presence of *Desulfobacter*-like organisms in the ^{13}C-acetate slurry (Table 2; Fig. 4). Genes of *dsrA* belonging to the *Desulfobacteraceae* dominated the ^{13}C-DNA library, which included *Desulfobacter*-, *Desulfosarcina*-like and *dsrA* group I (Thomsen *et al*., 2001) sequences that cluster with *Ds. multivorans* (Fig. 4). Other ^{13}C-DNA *dsrA* sequences belonged to *Syntrophobacteraceae* and *dsrA* groups III and IV (Thomsen *et al*., 2001; Dhillon *et al*., 2003), but no *Desulfotomaculum* sequences. *Syntrophobacteraceae* includes acetate-utilizing SRB, such as *Desulfovirga*, *Desulforhabdus* and *Desulfacinum* spp. (Rees *et al*., 1995; Tanaka *et al*., 2000). The PLFA of *Desulforhabdus amnigenus* are also dominated by odd-chain fatty acids with high amounts of C15 and C17 compounds (Rütters, 2001), similar to the ^{13}C-PLFA from the ^{13}C-acetate, ^{13}C-pyruvate slurries and the later ^{13}C-glucose slurry. However, *D. amnigenus* cannot utilize glucose and would have to rely on glucose breakdown products (e.g. pyruvate, ethanol and acetate), which may explain the change in the ^{13}C-glucose PLFA labelling pattern with time and its similarities with the ^{13}C-pyruvate and ^{13}C-acetate slurries.

Uncultivated *Actinobacteria*, *Firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Acidobacteria* sequences (Table 2) were also found in the ^{13}C-acetate slurry, suggesting that a broad range of organisms were active and able to utilize or incorporate acetate directly, or in close syntrophic association with acetate-utilizers. All of these phylogenetic groups have been previously reported to be in sedimentary environments. *Actinobacteria* and *Firmicutes* have been found in sulfur-rich environments and/or involved in sulfur cycling in sediments (Boschker *et al*., 1998; Elshahed *et al*., 2003; Goorissen *et al*., 2003). Cultivated sulfur or thiosulfate oxidizers able to grow on acetate are found in the *Alpha*- and *Gammaproteobacteria*, and within members of the *Bacteroidetes* (Teske *et al*., 2000). Whereas, most members of the *Acidobacteria* are, as yet, uncultivated but they have been detected by molecular techniques in sediments and submarine vents (Sievert *et al*., 2000; Bowman and McCuaig, 2003). Candidates for novel sulfate reducers are not limited to taxa classically associated with this process (Loy *et al*., 2002). It is possible that some of the unidentified *dsrA* genes in the ^{13}C-DNA of the acetate slurry (Fig. 4) belong to uncultured groups of SRB within the *Firmicutes*, *Actinobacteria*, *Acidobacteria* or others such as JS1 (Tables 1 and 2).

Polymerase chain reaction-DGGE analysis of bacterial 16S rRNA genes revealed that the dominant sequences in the ^{13}C-acetate slurry belonged to uncultured members of the *Firmicutes* and the candidate division JS1 (Table 1). The presence of JS1 in the ^{13}C-acetate-DNA fraction was further confirmed (Fig. 3E) by the use of specific JS1 primers (Webster *et al*., 2004) suggesting that these organisms play a role in acetate-metabolizing sulfate-reducing sediments. This is the first direct evidence for the metabolism of this uncultured bacterial group; previous studies have only inferred physiological properties. For example, JS1 have been identified in sulfate-reducing consortia able to mineralize benzene (Phelps *et al*., 1998), in methane hydrate bearing sediments (Reed *et al*., 2002), associated with anaerobic methanotrophic communities in sediments with high sulfate reduction rates (Teske *et al*., 2002; Dhillon *et al*., 2003), and in methane- and hydrocarbon-discharging, brine-soaked, mud breccias in a terrestrial mud volcano (Alain *et al*., 2006). In this study we have directly confirmed that JS1 is present and active under anaerobic sulfate-reducing conditions and able to utilize glucose (or glucose metabolites) and acetate, which may provide an insight into the ecology of this organism. In addition, members of JS1 might have odd, branched PLFAs similar to those that have been associated with sulfate-reducing populations in marine sediments (Pancost *et al*., 2000).

The differences in the dominant bacterial taxa between the two 16S rRNA gene approaches for the analysis of the ^{13}C-acetate slurry might be explained by differences in specificity of the PCR primers used. It has been reported that different PCR primers target different phylogentic groups (e.g. Marchesi *et al*., 1998; Webster *et al*., 2006), and it may be that the approach used in this study, with multiple sets of PCR primers, has allowed a more comprehensive assessment of the active bacterial community.

In conclusion, this study has demonstrated that the combined use of DNA- and PLFA-SIP can contribute to understanding carbon flow, substrate utilization and population structure of an interacting, anaerobic prokaryotic community. Different bacterial populations were highlighted that seemed to utilize glucose compared with those populations utilizing acetate, pyruvate and glucose metabolites. The identification of *Desulfococcus* and *Desulfosarcina* from ^{13}C-labelled PLFA and *dsrA* directly seemed to link these to acetate utilization, and ^{13}C-DNA further indicated that the newly identified bacterial division JS1 and *Firmicutes* were active in sediment slurries. However, the interpretation of combined biomarker-SIP data is complex and presents both challenges and new opportunities for future SIP studies.

Experimental procedures

Pure cultures

Pseudomonas putida strain UWC1 was grown in SBS mineral medium (Slater *et al*., 1979) supplemented with 0.1% (w/v) ^{13}C-glucose (U-$^{13}C_6$, 99%; CK Gas Products). *Mycobacte-*

rium smegmatis strain MC2155 was grown in nutrient broth with no ^{13}C-substrate addition. *Desulfobacter* sp. DSM 2035 was grown on DSM medium 195 supplemented with either ^{12}C-sodium acetate or ^{13}C-sodium acetate (1,2-^{13}C2, 99%; CK Gas Products).

Sediment slurry microcosms

Estuarine sediment cores (diameter 15 cm, depth 35 cm) were collected at low tide from tidal flats of the Tamar estuary, UK (50°21.25′N, 4°11.25′W) in November 2001. Sediment from the black sulfide-rich zone (1–10 cm depth) was used to establish replicate anaerobic sediment slurries. Sediment (250 ml) was pre-enriched in 750 ml of anaerobic mineral salts medium (Wellsbury *et al*., 1994) supplemented with 10 mM glucose, 10 mM sodium acetate, 18 mM sodium sulfate and 1 mM sodium sulfide contained within modified 1 l screw-capped Erlenmeyer flasks fitted with three-way stopcocks for sampling. The gas headspace was replaced with oxygen-free nitrogen. Flasks were incubated at 25°C in the dark on an orbital shaker (150 r.p.m.) until a stable state of acetate and sulfate decrease and sulfide increase was observed. Under these conditions of stimulated prokaryotic sulfate-reduction, labelled ^{13}C-substrates were added to a final concentration of 100 μM, and incubated with ^{13}C-glucose (U-$^{13}C_6$, 99%), ^{13}C-acetate (1,2-^{13}C2, 99%) or ^{13}C-pyruvate (U-$^{13}C_3$, 99%; CK Gas Products) for further 7 days.

DNA extraction

Genomic DNA was extracted from all pure cultures of organisms using the FastDNA kit (Qbiogene). Sediment DNA was extracted from 5 × 1 g ^{13}C-enriched sediment slurry using the Fast DNA spin kit for soil (Qbiogene) with modifications and pooled as described by Webster and colleagues (2003). DNA extracts were visualized by standard agarose gel electrophoresis, and the DNA quantified against Hyperladder I DNA marker (Bioline) using the Gene Genius Bio Imaging System (Syngene).

CsCl density gradient ultracentrifugation

The CsCl density gradient and DNA fractionation conditions were initially optimized using ~10 μg of ^{13}C-labelled DNA from *P. putida* and ~10 μg of ^{12}C-labelled DNA from *M. smegmatis.* Ethidium bromide (100 μg ml^{-1} DNA solution; Qbiogene) and CsCl (1 g ml^{-1} DNA solution; Sigma) was added to the DNA solution, and the resulting solution transferred to a Quick–Seal pollyallomer ultracentrifuge tube (~13.5 ml; 16 × 76 mm; Beckman Coulter UK). DNA fractions were resolved following ultracentrifugation at 265 000 *g* (55 000 r.p.m.) using a VTi 65.1 vertical-tube rotor (Beckman Coulter UK) for 16 h at 20°C in a CsCl density gradient (Radajewski *et al*., 2000). The ^{12}C- (*M. smegmatis*) and ^{13}C-DNA (*P. putida*) fractions (< 0.5 ml) were carefully removed from the CsCl gradient tube with a 19-gauge hypodermic needle and collected into 1.5 ml tubes. DNA fractions were then extracted with sterile water-saturated 1-butanol three times and dialysed in a Microcon YM100 filter device (Millipore Corporation) with molecular grade water (MGW) (Severn Biotech) to remove ethidium bromide and CsCl contamination. Purified DNA fractions were finally eluted in 50 μl of MGW and stored at −20°C until analysis.

DNA (~5 μg) from the ^{13}C-enriched sediment slurries was fractionated as described above. However, because of very low concentrations of ^{13}C-DNA, and therefore no visible ^{13}C band within the gradient tube, ^{12}C- and ^{13}C-DNA fractions were removed alongside a 'marker' tube containing *Desulfobacter* sp. DSM 2035 ^{12}C- and ^{13}C-DNA (~5 μg) as a visual guide (see Fig. 3). DNA fractions were then cleaned as described above and eluted in 30 μl of MGW.

16S rRNA gene PCR-DGGE analysis

Bacterial 16S rRNA genes were amplified from sediment slurry DNA extracts with primers 357F-GC and 518R (Muyzer *et al*., 1993) as previously described (Webster *et al*., 2003). Polymerase chain reaction amplimers were analysed by DGGE as described (Webster *et al*., 2003) using the DCode system (Bio-Rad) on 8% (w/v) polyacrylamide gels with a denaturant gradient between 30% and 60% [100% denaturant equals 7 M urea and 40% (v/v) formamide]. Gels were stained with SYBRGold nucleic acid stain (Molecular Probes), viewed under UV and images captured using a Gene Genius Bio Imaging System (Syngene). Denaturing gradient gel electrophoresis bands of interest were excised, re-amplified by PCR and sequenced as described (Webster *et al*., 2003).

Polymerase chain reaction amplification of bacterial and JS1 candidate division 16S rRNA genes and cloning

Bacterial and JS1 candidate division 16S rRNA genes were amplified from the ^{13}C-acetate-enriched sediment slurry ^{13}C-DNA fraction using PCR primers 27F-907R (Lane, 1991; Muyzer *et al*., 1998) and 63F-665R (Marchesi *et al*., 1998; Webster *et al*., 2004), respectively, and PCR conditions were as described (Newberry *et al*., 2004; Webster *et al*., 2004). Five PCRs were pooled, cleaned and concentrated using Microcon YM100 spin filters (Millipore Corporation) and eluted in 40 μl of sterile distilled water. Purified products were quantified and ligated into pGEM T-easy vector, and transformed into *Escherichia coli* JM109 competent cells (Promega Corporation). Clones containing the correct insert, after re-amplification with M13 primers were sequenced in an ABI PRISM 3100-Genetic Analyzer (Applied Bio-systems). Additionally, JS1 PCR products from both the ^{12}C- and ^{13}C-DNA fractions taken from the ^{13}C-acetate-enriched sediment slurry were also analysed by a nested PCR-DGGE approach (Webster *et al*., 2004).

Polymerase chain reaction amplification of dsrA *genes, cloning and phylogenetic analysis*

Dissimilatory sulfite reductase (*dsrA*) gene sequences were amplified from the acetate-enriched sediment ^{12}C- and ^{13}C-DNA fractions using the PCR primers DSR1F and DSR4R (Wagner *et al*., 1998). Amplifications were performed with 0.2 pmol μl^{-1} of primers, 1 μl of DNA template, 1× reaction buffer (Bioline), 1.5 mM $MgCl_2$, 2.0 U Biotaq DNA poly-

merase (Bioline), 0.125 mM each dNTP, 10 μg of bovine serum albumin (Promega Corporation) in a 50 μl PCR reaction mixture with MGW (Severn Biotech). Controls with 1 μl of MGW and 1 μl of DNA from *Desulfobacter* sp. DSM 2035 were included in all sets of PCRs undertaken. Reactions were carried out at 95°C for 2 min followed by 40 cycles of 94°C for 30 s, 54°C for 45 s and 72°C for 90 s, with a final extension step at 72°C for 7 min in a DNA Engine Dyad Thermal Cycler (MJ Research). Five PCRs from each sample were pooled and the PCR products electrophoresed on a 1.2% (w/v) agarose gel stained with ethidium bromide. DNA bands of correct size were cut out and the DNA extracted using Quantum Prep Freeze 'N Squeeze™ Spin Columns (Bio-Rad). Gel extracted DNA was purified and concentrated using Microcon YM 100 spin filters (Millipore Corporation) and eluted in 40 μl of sterile distilled water, quantified and ligated into pGEM T-easy vector, and transformed into *E. coli* JM109 competent cells (Promega Corporation). Clones containing the correct insert, after re-amplification with primers DSR1F and DSR4R, had their first 600 bp sequenced two times with the DSR1F primer in an ABI PRISM 3100-Genetic Analyzer (Applied Bio-systems). Clones were analysed by rarefaction (Kemp and Aller, 2004) to determine the extent to which the number of clones was sufficient to represent the diversity in *dsrA* clone libraries.

Sequence analysis

Sequence chromatographs were analysed using the Chromas software package version 1.45 (http://www.technelysium.com/au/chromas.html). Partial sequences and their closest relatives were identified by NCBI BLAST (http://www.ncbi.nlm.nih.gov/). All nucleotide sequences were aligned using CLUSTALX (Thompson *et al*., 1997) with sequences retrieved from the database. Alignments were edited manually using BioEdit Sequence Alignment Editor version 5.0.9 (Hall, 1999) and regions of ambiguous alignment removed. Guide trees were constructed using neighbour-joining with the Jukes and Cantor correction algorithm in MEGA version 2.1 (Kumar *et al*., 2001). However, phylogenetic relationships between pairs of nucleotide sequences were calculated using LogDet/Paralinear distances of variable sites as the primary tool and implemented in PAUP*4.0b 10 (Swofford, 1998). The maximum-likelihood method was used to estimate the proportion of invariable sites (Webster *et al*., 2004). All LogDet/Paralinear distances trees were constructed by using the minimum evolution criterion and the data bootstrapped 1000 times to assess support for nodes. Additionally, phylogenetic analysis also included LogDet/Paralinear distances with codon position 3 excluded to compensate for potential mutational saturation and nucleotide base composition heterogeneity (Horner *et al*., 2000), as well as maximum parsimony on translated amino acid sequences using PAUP*4.0b 10. All treeing methods resulted in similar tree topologies.

The new sequences reported here have been submitted to the database under accession numbers AM117992–AM118017 and AM162284–AM162293 for 16S rRNA gene sequences and AM117951–AM117991 for *dsrA* gene sequences.

Chemical analysis of bacterial activity and direct bacterial enumeration

Sulfide concentrations were determined spectrophotometrically by the methylene blue method (Cline, 1969). Sulfate concentrations were determined by Ion Exchange Liquid Chromatography (Dionex) using an AS4A anion-exchange column with AG4A guard column coupled to a micromembrane suppresser and conductivity detector. Total bacterial numbers were determined by acridine orange direct counts as previously described (Parkes *et al*., 2005).

Lipid extraction, fractionation, derivatization and gas chromatographic determination

Lipids were extracted using a modified Bligh-Dyer method (Rütters *et al*., 2002) from 1 to 5 g of dry weight freeze-dried sediment. Total lipid extracts were fractionated into neutral lipids, free fatty acids (FFA) and polar lipids (including phospholipids) using an aminopropyl bond elut solid-phase extraction cartridge with 100 mg sorbent mass and 1 ml capacity. After conditioning/pre-extracting of the cartridge with 12 ml of n-hexane, the lipid extract was dissolved in 0.5 ml of 2:1 (v:v) dichloromethane:isopropanol (0.5 ml) and added to the top of the sorbent. Fractions were eluted sequentially with 7 ml of 2:1 (v:v) dichloromethane:isopropanol (neutral lipids), 7 ml of 2% glacial acetic acid in diethylether (FFA) and 7 ml of methanol (polar lipids). The fractions were dried under a stream of nitrogen and stored at –20°C. The polar lipid fraction containing PLFA was transesterified with 1 ml of 14% BF_3 in methanol solution, of known ^{13}C isotopic composition, at 50°C for 1 h. If necessary, structural identification of PLFA was improved by dimethyl disulfide and picolinyl ester derivatization (Christie, 1998) followed by gas chromatography-mass spectrometry (GC-MS) analysis.

The methyl esters were analysed with a gas chromatograph equipped with a flame ionisation detector (GC-FID; Varian 3500) on a Supelco Omegawax 320 column (polyethylene glycol phase, 30 m × 0.32 mm i.d., 0.25 μm film). Hydrogen was used as carrier gas, and the temperature of the oven was programmed as follows: 50–100°C at 10°C min^{-1}, 100–240°C at 4°C min^{-1} (20 min isothermal). Quantification of each lipid was calculated using the peak areas, and by comparison with the known amount of a co-injected standard (C12 FAME or C19 alkane). Tentative identification based on retention time was confirmed on a GC-MS (Carlo Erba 5160) equipped with a Chromapack CPWax-52 CB column (50 m × 0.32 mm i.d., 0.12 μm film). Helium was used as carrier gas (10 psi head pressure), and the GC was coupled via a heated transfer line (320°C) to a Finnigan MAT 4500 quadropole mass spectrometer scanning in the range of 50–650 m/z with a cycle time of 1 s. The same temperature program as for the GC-FID experiments was used. Carbon isotopic composition of PLFA was determined on a Varian 3500 gas chromatograph equipped with a ZB-Wax column (polyethylene glycol phase, 30 m × 0.32 mm i.d., 0.12 μm film) coupled via Finnigan type I combustion interface to a Finnigan MAT Delta-S isotope ratio mass spectrometer. Helium was the carrier gas (15 psi head pressure), and oven temperature was programmed as follows: 40°C (1 min isothermal), 40–100°C at 10°C min^{-1}, 100–240°C

at 4°C min^{-1} (20 min isothermal). The carbon isotopic composition was corrected for the exogenous derivative methyl group (Rieley, 1994).

Phospholipid fatty acid nomenclature

The nomenclature for the PLFA is in the form A:BωC, where A designates the total number of carbons, B the number of double bonds and C the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes -c (*cis*) and -t (*trans*) refer to geometric isomers and the prefixes *i*- (iso) and *a*- (anteiso) refer to methyl branching (Ratledge and Wilkinson, 1988), and br to unresolved branching.

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