RESEARCH ARTICLE

Enrichment and cultivation of prokaryotes associated with the sulphate–methane transition zone of diffusion-controlled sediments of Aarhus Bay, Denmark, under heterotrophic conditions

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Abstract

The prokaryotic activity, diversity and culturability of diffusion-controlled Aarhus Bay sediments, including the sulphate–methane transition zone (SMTZ), were determined using a combination of geochemical, molecular (16S rRNA and *mcrA* genes) and cultivation techniques. The SMTZ had elevated sulphate reduction and anaerobic oxidation of methane, and enhanced cell numbers, but no active methanogenesis. The prokaryotic population was similar to that in other SMTZs, with *Deltaproteobacteria*, *Gammaproteobacteria*, JS1, *Planctomycetes*, *Chloroflexi*, ANME-1, MBG-D and MCG. Many of these groups were maintained in a heterotrophic (10 mM glucose, acetate), sediment slurry with periodic low sulphate and acetate additions (~2 mM). Other prokaryotes were also enriched including methanogens, *Firmicutes*, *Bacteroidetes*, *Synergistetes* and TM6. This slurry was then inoculated into a matrix of substrate and sulphate concentrations for further selective enrichment. The results demonstrated that important SMTZ bacteria can be maintained in a long-term, anaerobic culture under specific conditions. For example, JS1 grew in a mixed culture with acetate or acetate/glucose plus sulphate. *Chloroflexi* occurred in a mixed culture, including in the presence of acetate, which had previously not been shown to be a *Chloroflexi* subphylum I substrate, and was more dominant in a medium with seawater salt concentrations. In contrast, archaeal diversity was reduced and limited to the orders *Methanosarcinales* and *Methanomicrobiales*. These results provide information about the physiology of a range of SMTZ prokaryotes and shows that many can be maintained and enriched under heterotrophic conditions, including those with few or no cultivated representatives.

Introduction

The sulphate–methane transition zone (SMTZ; Reeburgh, 2007), also referred to as the sulphate–methane interface (e.g. Kasten & Jørgensen, 2000) or the sulphate–methane reaction zone (e.g. Halbach *et al.*, 2004), is defined as the horizon within marine sediments where sulphate and methane coexist (Iversen & Jørgensen, 1985; Treude *et al.*, 2005). The SMTZ is a general feature of marine sediments representing a diffusion-controlled interface associated with enhanced prokaryotic activity (Parkes *et al.*, 2005). The SMTZ is located between an upper zone, where sulphate reduction is the dominant prokaryotic respiration process, and a lower zone, where methanogenesis becomes dominant. Within the SMTZ, most sulphate removal is presumed to be directly coupled to anaerobic oxidation of methane (AOM; Knittel & Boetius, 2009) carried out by a syntrophic partnership between uncultivated lineages of sulphate-reducing bacteria (SRB), belonging to the *Deltaproteobacteria*, and anaerobic methane-oxidizing *Archaea* (ANME; Boetius *et al.*, 2000). Considerable advances have been made in understanding the biology of microorganisms directly

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involved in AOM by applying a range of culture-independent methods such as ^{13}C-lipid biomarker analysis, 16S rRNA gene surveys, FISH and metagenomics (Hinrichs *et al.*, 1999; Boetius *et al.*, 2000; Knittel *et al.*, 2005; Meyerdierks *et al.*, 2005, 2009; Lösekann *et al.*, 2007). However, very little is known about the diversity, role and association with AOM of the other prokaryotes present within the SMTZ. This is despite the total prokaryotic populations being shown to increase at SMTZs (Fry *et al.*, 2008) and that other prokaryotes not typically associated with AOM seem to be the majority, as population densities of ANME at SMTZs are remarkably low (Knittel & Boetius, 2009) and SRB populations may be only ~22% of the total SMTZ population (Leloup *et al.*, 2009).

Harrison *et al.* (2009), studying the microbial assemblage of the SMTZ of sediments from the Santa Barbara Basin, showed by statistical comparisons with data from other studies of prokaryotic diversity in SMTZ, hydrate-bearing, methane seeps and organic matter-rich marine sediments that other bacterial groups not typically associated with AOM are also enriched in the SMTZ. For example, *Bacteria* belonging to the phylum *Planctomycetes* and the candidate division JS1 (Webster *et al.*, 2004), and large numbers of *Chloroflexi* sequences dominate 16S rRNA gene libraries from sediments directly below the SMTZ, suggesting that these groups may be important members of the SMTZ prokaryotic community (Harrison *et al.*, 2009). Other studies show that the same taxa are dominant within methane hydrate-bearing sediments (Inagaki *et al.*, 2006) and in subsurface tidal flat sediments with low concentrations of sulphate and high concentrations of recalcitrant carbon (Webster *et al.*, 2007). In addition, novel groups of *Archaea*, not known to play a role in AOM, are frequently identified within SMTZ and methane-containing sediments. For example, *Crenarchaeota* Marine Benthic Group B (MBG-B; Vetriani *et al.*, 1999) are present in the SMTZ of Peru Margin (Biddle *et al.*, 2006; Sørensen & Teske, 2006) and Santa Barbara Basin (Harrison *et al.*, 2009), and gassy sediments of Marennes-Oléron Bay (Roussel *et al.*, 2009). Also, *Euryarchaeota* MBG-D related to *Thermoplasmatales* are found in methane hydrate-bearing sediments of the Pacific Ocean (Inagaki *et al.*, 2006). However, despite such a diverse range of prokaryotes being dominant in marine sediment SMTZ and related sites, representatives of the majority of these dominant bacterial and archaeal taxa have not yet been obtained by laboratory cultivation, and hence, little is known about their metabolism or physiology.

Conventional cultivation of marine sediment prokaryotes is time consuming and often selects for the growth of unrepresentative bacteria (Fry *et al.*, 2008). Here, we describe the use of a routinely monitored heterotrophic sediment slurry enrichment made from geochemically and microbially characterized diffusion-controlled SMTZ sediments of Aarhus Bay, Denmark, for the selective enrichment of SMTZ prokaryotes. Because Aarhus Bay sediments receive regular inputs of organic carbon from primary production during the summer months (Glud *et al.*, 2003), it was considered that the addition of organic substrates to an SMTZ sediment slurry maintained under low-sulphate conditions (concentrations within the SMTZ) would stimulate heterotrophic prokaryotic activity, resulting in the production of further substrates and conditions for the enrichment of SMTZ prokaryotes including sulphate reducers, methanogens, ANME and other representative types.

The diversity of *Bacteria* and *Archaea* and prokaryotic activity were monitored in this heterotrophic sediment slurry enrichment using a combination of molecular [PCR-denaturing gradient gel electrophoresis (DGGE) and sequencing of 16S rRNA and *mcrA* genes] and geochemical analyses. After 6 months of enrichment culture and the production of a complex, stable and representative SMTZ prokaryotic community, further selective enrichment and isolation of organisms, in an attempt to select for representatives of the major bacterial groups of JS1 and *Chloroflexi*, was carried out in 96-deep-well plates containing a matrix of different concentrations of sulphate, acetate and glucose.

Materials and methods

Sediment sampling

Aarhus Bay is a shallow semi-enclosed embayment on the transition between the North Sea and Baltic Sea, characterized by elevated primary production during the summer months (Glud *et al.*, 2003). The sediments are 6–7-m-thick Holocene mud deposits that overlay brackish, late glacial clay-silt and till, and free methane gas is widespread (Laier & Jensen, 2007). Core (12 cm diameter, 3 m length) samples of sediment from Aarhus Bay station M5 were taken on 13 December 2004 using a gravity corer during the METROL Aarhus Bay cruise HN04F on the St F. Henry for biogeochemical (165GC, 56°6.204′N 10°27.468′E) and microbiological analysis (167GC, 56°6.201′N 10°27.467′E; 169GC 56°6.202′N 10°27.467′E). Sampling for biogeochemical analysis was carried out immediately as described (Dale *et al.*, 2008). The uppermost 20 cm of sediment was lost during gravity coring (Dale *et al.*, 2008), and therefore, the depth data presented in this study were corrected to show true sediment depth.

Sediment cores for microbiological analysis were cut into 1-m section, and 3-cm^3 sample were taken immediately at various depths from each section for methane analysis to identify the SMTZ. Subsequently, the top 1 m below seafloor (mbsf) section, containing the SMTZ, was cut into 0.5-m section, capped and sealed in gas-tight aluminium bags

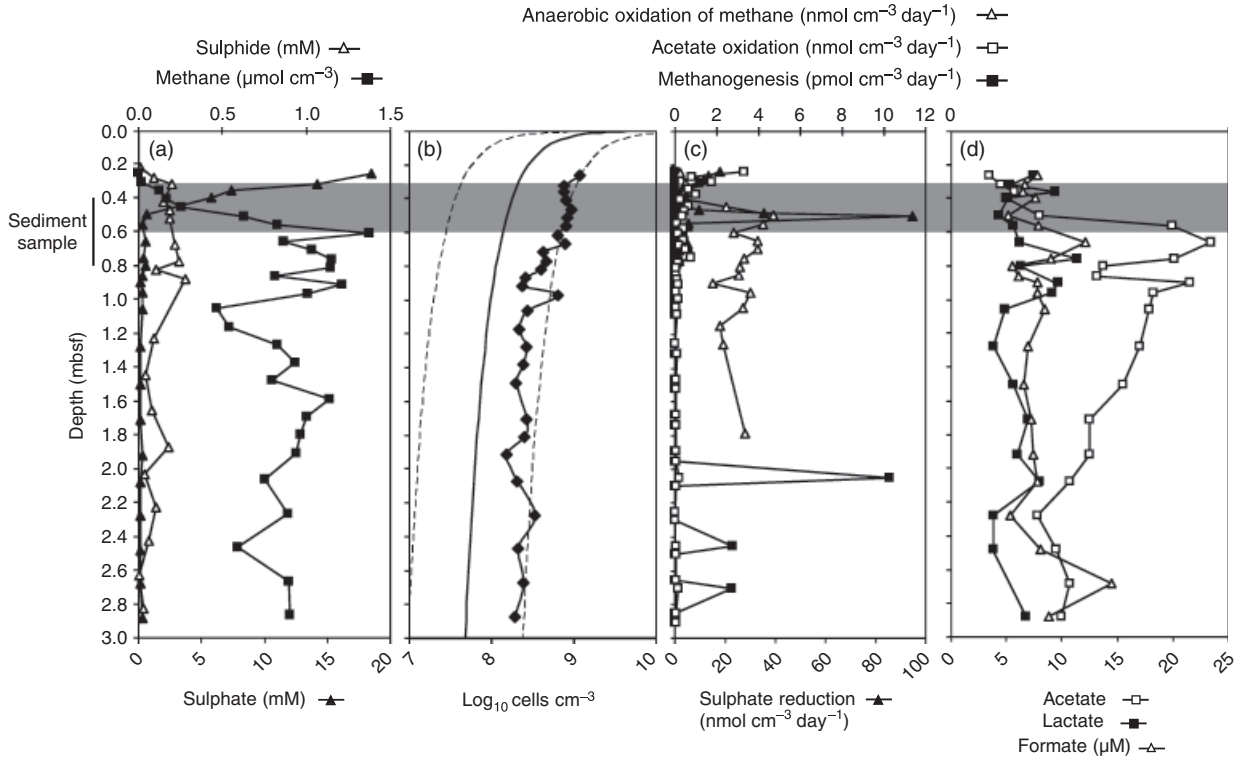

Fig. 1. Depth profiles of geochemical data, prokaryotic cell numbers and prokaryotic activity for Aarhus Bay sediment core 165GC. (a) Pore water sulphate, sulphide and methane (Fossing, 2005) concentrations. (b) Prokaryotic cell numbers (AODC). The solid line shows the Parkes *et al*. (2000) general model for prokaryotic cell distributions in marine sediments, and the dotted lines represent 95% prediction limits. (c) Potential rates of sulphate reduction (Fossing, 2005), acetate oxidation to CO_2, AOM, and methanogenesis from acetate and H_2/CO_2 (note that all measured rates of H_2/CO_2 methanogenesis were zero). (d) Pore water acetate, lactate and formate concentrations. The shaded region denotes SMTZ at ~0.3–0.6 mbsf. Sediment sample depth used to prepare the SMTZ sediment slurry enrichment is also shown.

under anaerobic conditions with a nitrogen atmosphere and an Anaerocult A (Merck), and stored at 4 °C. Stored sediment cores were then further subsampled (19 May 2005) under aseptic and anaerobic conditions in the laboratory (Parkes *et al.*, 1995), and the ~0.4–0.8 mbsf section (including the SMTZ; Fig. 1) from each core was pooled and mixed in an anaerobic chamber and then used as an inoculum for an SMTZ sediment slurry enrichment.

Acridine orange direct counts (AODC) and prokaryotic activity measurements

Sediment samples (from core 165GC) for AODC were fixed immediately after sampling in 2% w/v formaldehyde containing 3.5% w/v NaCl and counted as described by Fry (1988).

Samples for measuring methanogenesis and acetate oxidation rates were taken immediately after sampling (Parkes *et al.*, 1995) using 10-cm-long minicores (2 cm diameter) fitted with sealant-filled injection ports at 1-cm interval, with sterile butyl-rubber stoppers, and left overnight to equilibrate at *in situ* temperature. Prokaryotic activity (acetoclastic methanogenesis, acetate oxidation and hydrogenotrophic methanogenesis) was measured by injecting each minicore two or three times with [1,2-^{14}C]-acetic acid (sodium salt; 2 μL, 43.8 kBq; Amersham Biosciences) or sodium [^{14}C]-bicarbonate (2 μL, 38.6 kBq; Amersham Biosciences) and incubated at *in situ* temperature for 7 and 18 h, respectively. After incubation, the sediment in the minicores was extruded using a sterile plunger, and 2 cm of the sediment column around each injection site was removed and placed into 30-mL glass jar containing 7 mL of 2 M NaOH plus a magnetic stirrer, to terminate the incubation. The jars were capped with a butyl-rubber bung, agitated to disperse the sediment plug and stored inverted at room temperature until processing back in the laboratory.

For ^{14}C-CH_4 analysis, samples were magnetically stirred while the headspace gas was flushed for 20 min with 5% O_2: 95% N_2 and passed over copper oxide at 800 °C to convert ^{14}C-CH_4 to ^{14}C-CO_2. Flushed gases were bubbled through a series of three scintillation vials of 10 mL of Hi-Safe 3 scintillation cocktail (Canberra-Packard) containing 7% v/v β-phenethylamine to absorb any ^{14}C-CO_2. Scintillation vials were counted in a scintillation counter and label turnover

rates and potential activity rates were calculated as described (Parkes *et al.*, 2010).

Analysis of acetate oxidation was carried out after processing for acetoclastic methanogenesis, by transferring the slurry into a 120-mL round bottom flask and adding a sufficient quantity of 3.5 M HCl to neutralize the NaOH and to acidify the sample to enable the release of dissolved ^{14}C-CO_2; while stirring, the headspace was flushed for 20 min with nitrogen carrier gas into a series of three scintillation vials. Vials were counted as above and similar calculations were used to produce potential rate measurements.

The rates of sulphate reduction and anaerobic oxidation were measured as described by Dale *et al.* (2008), and some data (Fossing, 2005) were retrieved from the publishing network for Geoscientific and Environmental data system PANGAEA (http://www.pangaea.de/).

SMTZ sediment slurry enrichment

Pooled and mixed SMTZ sediment (250 mL) was added to 750 mL of anaerobic artificial marine medium (Shlimon *et al.*, 2004) supplemented with 2 mM sodium sulphate. The slurry was contained in a modified 2-L screw-capped bottle fitted with two shoulder ports for gas input, headspace sampling and supplement addition, and a three-way stopcock at the base for slurry sampling. Headspace gas was oxygen-free N_2 and the slurry was incubated at 25 °C in the dark on an orbital shaker (150 r.p.m.) after an initial addition of 10 mM glucose and 10 mM sodium acetate. Samples were taken regularly for gas analysis, aqueous geochemistry and molecular genetic analysis. Slurry conditions were maintained at ~2 mM sulphate with regular additions of sodium sulphate. In addition, after about 2 months, the slurry was supplemented with regular additions of 2 mM sodium acetate to prevent substrate limitation.

Chemical analysis of sediment slurry pore water and headspace gas

Pore water was obtained from sediment slurries by centrifugal extraction as described (Webster *et al.*, 2010). Sulphate, volatile fatty acid (VFA) concentrations and other anions were determined using an ICS-2000 Ion Chromatography System with an AS50 autosampler (Dionex UK Ltd) fitted with two Ionpac AS15 columns in series, and an Anion Self-Regenerating Suppressor (ASRS-ULTRA II 4 mm) in combination with a DS6 heated conductivity cell (Dionex UK Ltd) under the conditions described (Webster *et al.*, 2009).

For CH_4, H_2 and CO_2 measurements, headspace gas was removed and analysed immediately by GC, using a modified Perkin Elmer/Arnel Clarus 500 Natural Gas Analyser fitted with a flame ionization detector and a thermal conductivity detector.

Selective enrichment of SMTZ prokaryotes

After 6 months of incubation, the SMTZ sediment slurry was used as an inoculum for further enrichment of SMTZ sediment *Bacteria* and *Archaea*. Two polypropylene 96-deep-well plates (Beckman Coulter Inc.) containing a combination of concentrations of sulphate (0–5 mM), glucose and acetate (0, 0.1, 1 and 10 mM) were inoculated with 40 μL of sediment slurry per well in an anaerobic chamber (see Supporting Information, Tables S1 and S2) and made up to 1 mL with anaerobic media. One plate was prepared with anaerobic artificial marine medium (medium 1) as described by Shlimon *et al.* (2004), and the other with a slightly higher salinity and more complex anaerobic artificial seawater (medium 2) described by Köpke *et al.* (2005). Both plates were incubated at 25 °C for 3 months, analysed for growth and subcultured into replicate 96-deep-well plates, and incubated for a further 3 months. Deep-well plates were then analysed by fluorescence and PCR-DGGE analysis.

Estimation of cell numbers using SYBR Green I fluorescence

The growth and cell numbers in the 96-deep-well plate enrichments were determined as described (Martens-Habbena & Sass, 2006). Essentially, 200 μL of enrichment culture was mixed with 50 μL of 1 : 2000 working stock of SYBR Green I (Molecular Probes) diluted with TE buffer (200 mM Tris-HCl, 50 mM sodium EDTA, pH 8) in black 96-well microtitre plates (F96 MicroWell™ Plates; Nunc GmBH & Co. KG). Plates were then incubated in the dark at 25 °C for 4 h and the fluorescence was measured using a fluorescence microplate reader (Fluorocount BF10001, Packard BioScience) with excitation and emission wavelengths of 485 and 530 nm, respectively. To calibrate fluorescence with prokaryotic cell numbers, a mixture of marine subsurface bacterial isolates (*Desulfovibrio profundus* DSM 11384, *Acetobacterium* sp. Ac1 and *Photobacterium* sp. F18I) was prepared in equal ratios, counted using AODC and sequentially diluted to obtain a standard curve.

DNA extraction

DNA was extracted from all sediment and sediment slurry samples using the FastDNA® Spin Kit for Soil (MP Biomedicals) as described by Webster *et al.* (2003). DNA was also extracted from 96-deep-well plate enrichments using Chelex 100 (Walsh *et al.*, 1991; Parkes *et al.*, 2010). Essentially, 200 μL of each well was placed in a 96-well PCR plate (Bioline) and centrifuged at 2800 ***g*** for 10 min in a Hettich Rotanta 460R to pellet cells. Cell pellets were then resuspended in 50 μL of 5% w/v Chelex 100 (Sigma) and cells were lysed by heating to 98 °C in a DNA Engine Dyad Thermal Cycler (MJ Research) for 5 min, after which the

samples were placed on ice for a further 5 min. After a second heating and cooling step, the suspension was centrifuged as above to remove cell debris and the crude lysate was used directly for PCR-DGGE analysis. In addition, DNA was also extracted from several 96-deep-well plate enrichment samples (200 μL) using the FastDNA® Spin Kit for Soil to check for extraction bias. Identical DGGE patterns were obtained for DNA samples extracted using both methods.

16S rRNA and *mcrA* gene PCR-DGGE and 16S rRNA gene PCR cloning

Bacterial and archaeal 16S rRNA genes were amplified directly from DNA extracts of the sediment slurry and/or 96-deep-well plate enrichment samples with the bacterial primer pairs 357FGC-518R and the archaeal primers SAfGC-PARCH519R and analysed by DGGE (see Webster *et al.*, 2006a). Additionally, JS1 bacterial sequences were also targeted by nested PCR-DGGE with the primers 27F-665R and 357FGC-518R as described previously (Webster *et al.*, 2007). Methyl-coenzyme M reductase genes (*mcrA*) were amplified by nested PCR using the primers ME1f-ME2r (Hales *et al.*, 1996) and MLf-MLr (Luton *et al.*, 2002) without a GC-clamp and analysed on 6–12% gradient (w/v) polyacrylamide DGGE gels with a 25–50% denaturant gradient. All DGGE gels were stained with SYBR Gold nucleic acid stain (Molecular Probes), viewed under UV and images captured using a Gene Genius Bio Imaging System (Syngene). DGGE bands of interest were excised, reamplified by PCR, sequenced and analysed as described previously (O'Sullivan *et al.*, 2008).

Bacterial, JS1-targeted and archaeal 16S rRNA genes were also amplified directly from sediment slurry DNA extracts using the primers 27F-907R, 27F-665R and 109F-958R, respectively, and 16S rRNA gene libraries were made and analysed as described (Webster *et al.*, 2006b). It should be noted that the specificity of the JS1-targeted primer 665R is broad and matched 0.2% of the nontarget classified bacterial sequences in the RDP release 10 including *Chloroflexi*, *Proteobacteria*, *Firmicutes*, *Synergistetes* and *Acidobacteria*.

All new sequences reported here have been submitted to the EMBL database under accession numbers FR695317–FR695388 for 16S rRNA gene sequences and FR695389–FR695396 for *mcrA* gene sequences.

Statistical analysis of DGGE banding patterns

All representative DGGE band positions were excised and sequenced as above, and band identity was determined using the NCBI BLASTN algorithm (http://www.ncbi.nlm.nih.gov/). DGGE bands with different phylogenetic identities were given individual phylotype assignations and phylotype abundance was scored using a semi-quantitative scale relating to band intensity (dominant [brightest bands] = 5; present = 1; absent = 0). The data were analysed using the ordination methods: principal component analysis (PCA) and factor analysis using MINITAB Release 15 (Minitab Inc.) as described (Fry *et al.*, 2006). An initial PCA with all phylotypes included in the analysis showed that the first three components only explained around 22% of the variability, and therefore, subsequent analysis was undertaken with all rare phylotypes (analysis of individual plates, sum of scores ≤ 10; analysis of both plates combined, sum of scores ≤ 20) removed. The data matrix for the analysis used phylotype assignations as the variables, phylotype scores as the values within each variable and the correlation coefficient was used to calculate the similarity matrix. Groups identified by PCA and supported by factor analysis were analysed further by separate regression and correlation analysis to further support the main factors that might be affecting culturable bacterial diversity by correlating the phylotype scores with substrate and sulphate concentrations.

Results

Aarhus Bay sediment geochemistry and SMTZ prokaryotic activity

Pore water profiles, cell counts and prokaryotic activity measurements from core 165GC at station M5 are shown in Fig. 1. Sulphate concentrations decreased rapidly with depth from ~18 mM at the near surface (0.2 mbsf) to < 0.7 mM at 0.5 mbsf, while methane concentrations increased with depth (below 0.3 mbsf) and reached saturation at atmospheric pressure by 0.6 mbsf (Fig. 1a), demonstrating that the SMTZ was located between 0.3 and 0.6 mbsf. Prokaryotic cell numbers (AODC) throughout the core followed the global trend observed in other marine subsurface sediments (Parkes *et al.*, 2000) and decreased with depth following the upper prediction limit (Fig. 1b). Within the SMTZ, the cell numbers were relatively constant ($7.7–9.4 \times 10^8$ cells cm^{-3}), with a small peak in cell numbers at ~0.46 mbsf. Low concentrations (generally < 10 μM) of VFAs (acetate, lactate and formate) occurred throughout the SMTZ (Fig. 1d), with a peak in acetate (~23 μM) and other VFAs (lactate, 11.4 μM; formate, 12.1 μM) towards the base and below the SMTZ. Below the SMTZ, acetate decreased with depth while lactate and formate remained relatively constant, with the exception of a peak in formate at 2.7 mbsf (Fig. 1d).

The rates of sulphate reduction and AOM (mean rates = 17.3 and 2.2 nmol cm^{-3} day^{-1}, respectively, at 0.3–0.6 mbsf) were at their highest in and around the SMTZ, with peaks in sulphate reduction and AOM at ~0.5 mbsf (Fig. 1c). Acetate oxidation showed some activity in the SMTZ (mean

rates = 0.5 nmol cm^{-3} day^{-1}), but was the highest above the SMTZ at 0.2 mbsf. However, methanogenesis was not detected in the SMTZ (Fig. 1c), despite the presence of methane (Fig. 1a). Methanogenesis was only detected in deeper sediment depths below 2 mbsf, and then only acetoclastic methanogenesis occurred. This demonstrates that, although the methane in these sediments is biogenic, it diffuses up from depth towards the SMTZ (Dale *et al.*, 2009) and/or that there are other methanogenic substrates (Oremland *et al.*, 1982) present in and around the SMTZ that were not measured.

Aarhus Bay SMTZ prokaryotic community

Prokaryotic 16S rRNA gene libraries (Fig. 2) from the Aarhus Bay SMTZ showed that the prokaryotic population was composed of the bacterial taxa *Alpha-*, *Gamma-*, *Delta-*, *Epsilonproteobacteria*, *Chloroflexi*, *Planctomycetes*, candidate divisions JS1 and OP8, as well as *Archaea* belonging to the Miscellaneous Crenarchaeotic Group (MCG; Inagaki *et al.*, 2003), MBG-D/*Thermoplasmatales* groups, ANME-1 and other novel *Euryarchaeaota* (Fig. 2; Table S3). In summary, the majority of phylotypes were closely related to sequences belonging to uncultivated prokaryotes previously identified in marine sediments such as SMTZs (Harrison *et al.*, 2009), cold seeps (Orphan *et al.*, 2001) and subsurface sediments (Inagaki *et al.*, 2006), as well as cultivated members of the genera *Thiomicrospira*, *Colwellia* and *Halomonas* (Table S3). Interestingly, the absence of methanogen-related *Archaea* sequences is consistent with the absence of methanogenic activity in the SMTZ.

Aarhus Bay SMTZ sediment slurry enrichment

Prokaryotic activity

The addition of acetate and glucose to the SMTZ sediment slurry resulted in the immediate detection (Fig. 3a) of 9.1 mM acetate, which continued to increase due to glucose fermentation, until 5 days (13.6 mM). Acetate concentrations then fluctuated between 13 and 16 mM until ~40 days, when the concentrations started to decrease rapidly to 0.76 mM by 66 days, and then more slowly to 0.06 mM by 73 days. Similar trends were also detected for other VFAs such as formate, propionate and butyrate, but elevated

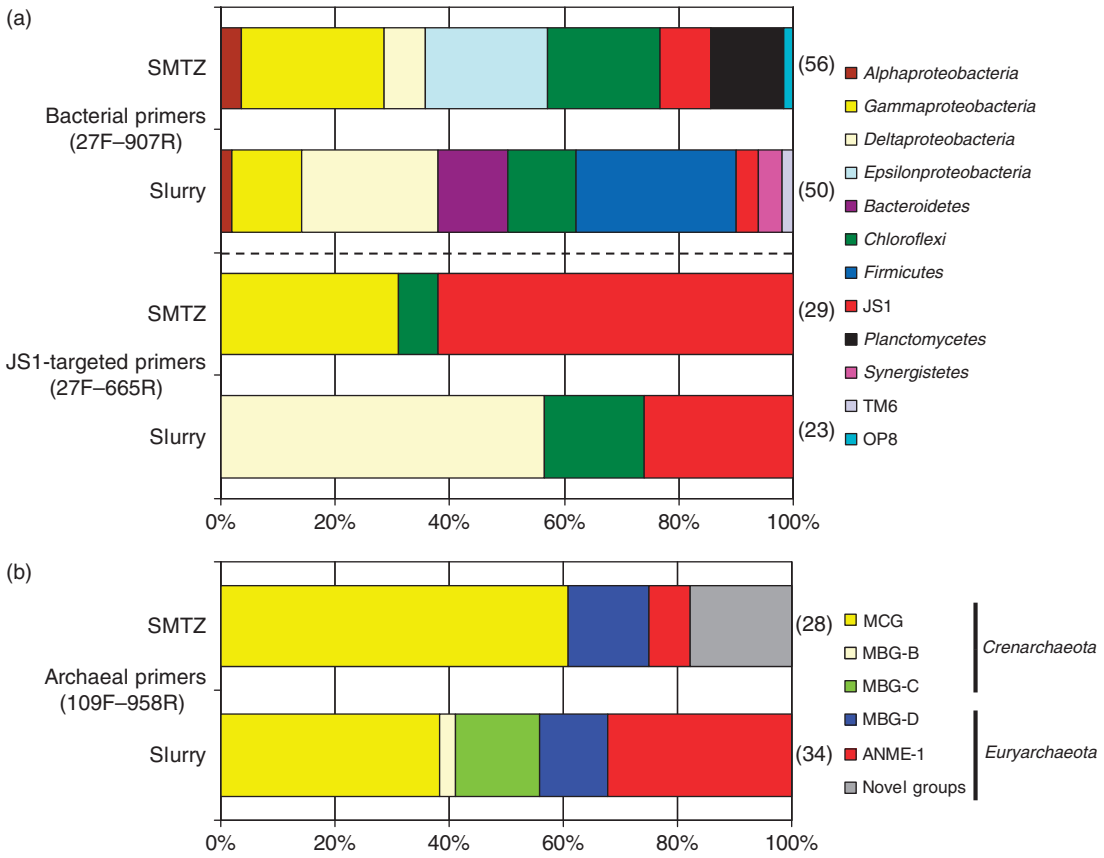

Fig. 2. Diversity of (a) bacterial and JS1-targeted, and (b) archaeal 16S rRNA gene sequences from Aarhus Bay SMTZ sediment (0.4–0.8 mbsf section – SMTZ) and Aarhus Bay SMTZ sediment slurry enrichment after 6 months of incubation (Slurry) derived by PCR cloning. The numbers of clones in each gene library are shown in parentheses.

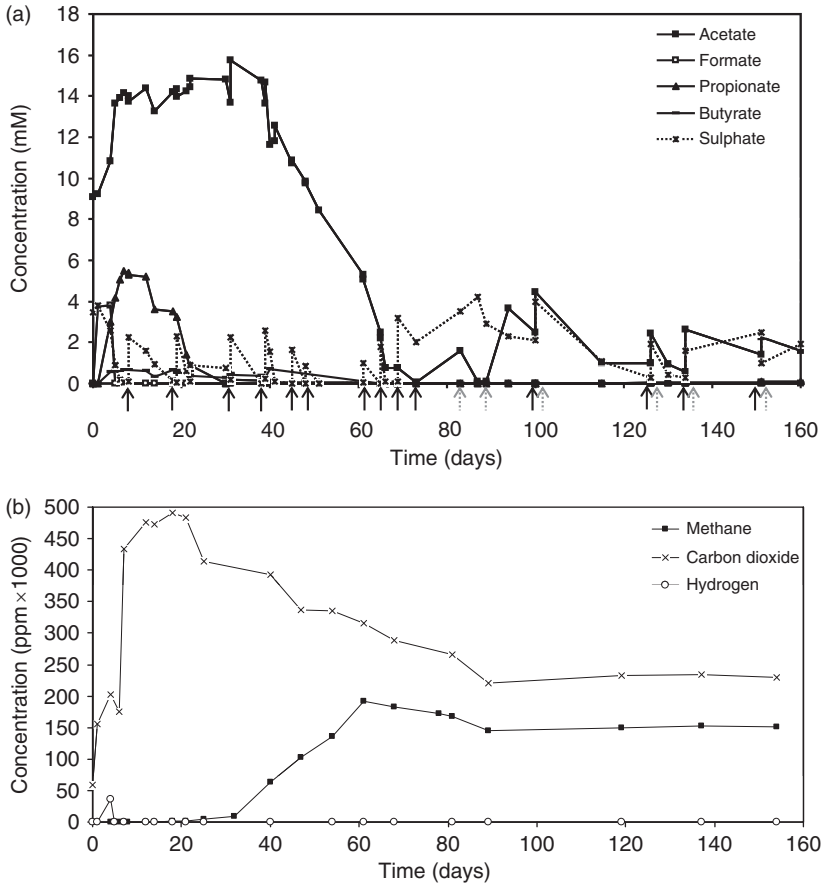

Fig. 3. Geochemical analysis of Aarhus Bay SMTZ sediment slurry enrichment incubated for up to 6 months. (a) Acetate, formate, propionate, butyrate and sulphate concentrations. Arrows denote the time points for the additions of sulphate (black) and acetate (grey). (b) Headspace methane, carbon dioxide and hydrogen concentrations.

concentrations were more transient and at much lower concentrations. Sequential utilization of VFA was coupled with continued sulphate removal (0.2–0.8 mmol L^{-1} day^{-1}) from 4 days, resulting in sulphate having to be continually replenished during the experiment (Fig. 3a). These sulphate reduction rates were ~10 × maximum rates in the SMTZ (95 nmol cm^{-3} day^{-1}; Fig. 1c), reflecting the enrichment and growth of the SRB population. In addition, rapid CO_2 production occurred during the first 5 days, which then remained constant until 25 days, before decreasing slowly (Fig. 3b). Methane production also occurred in the slurry after 5 days, with concentrations increasing rapidly after ~30 days (5750 p.p.m.v. CH_4 day^{-1}), which also coincided with acetate removal. A significant peak in hydrogen occurred by 4 days, which was rapidly consumed.

Prokaryotic community structure

The prokaryotic community in the SMTZ sediment slurry was periodically monitored throughout the 6-month enrichment using 16S rRNA gene PCR-DGGE. *Bacteria* (Fig. 4a) and JS1-targeted (Fig. 4b) PCR-DGGE demonstrated that there were some changes in the SMTZ sediment slurry community structure during the first 7–14 days of enrichment, with the emergence of a number of brightly stained bands. This enriched bacterial population then remained stable for the 6-month incubation period. Sequencing of excised DGGE bands (Fig. 4; Table 1) shows that the same taxa dominated the SMTZ bacterial community during enrichment from 14 days onwards, and were predominantly *Deltaproteobacteria* (*Desulfovibrio* related), *Gammaproteobacteria* (e.g. *Vibrio* and *Thiomicrospira* related) and JS1 (Fig. 4; Table 1). However, some bacteria, such as an *Epsilonproteobacteria* phylotype (band 4B; Fig. 4a), appeared only transiently. The appearance of *Vibrio*-related species within the sediment slurry may not be surprising as these bacteria are fast-growing, heterotrophic, facultative anaerobes that are often isolated from marine environments and rapidly utilize glucose (Alonso & Pernthaler, 2005) in sediment slurries (Webster *et al.*, 2010).

Further analysis of the bacterial population by PCR cloning after 6 months of incubation (Fig. 2a; Table S3) also demonstrated some change in the SMTZ bacterial population during enrichment, although the majority (62%) of the bacterial phyla remained the same. For example, the SMTZ

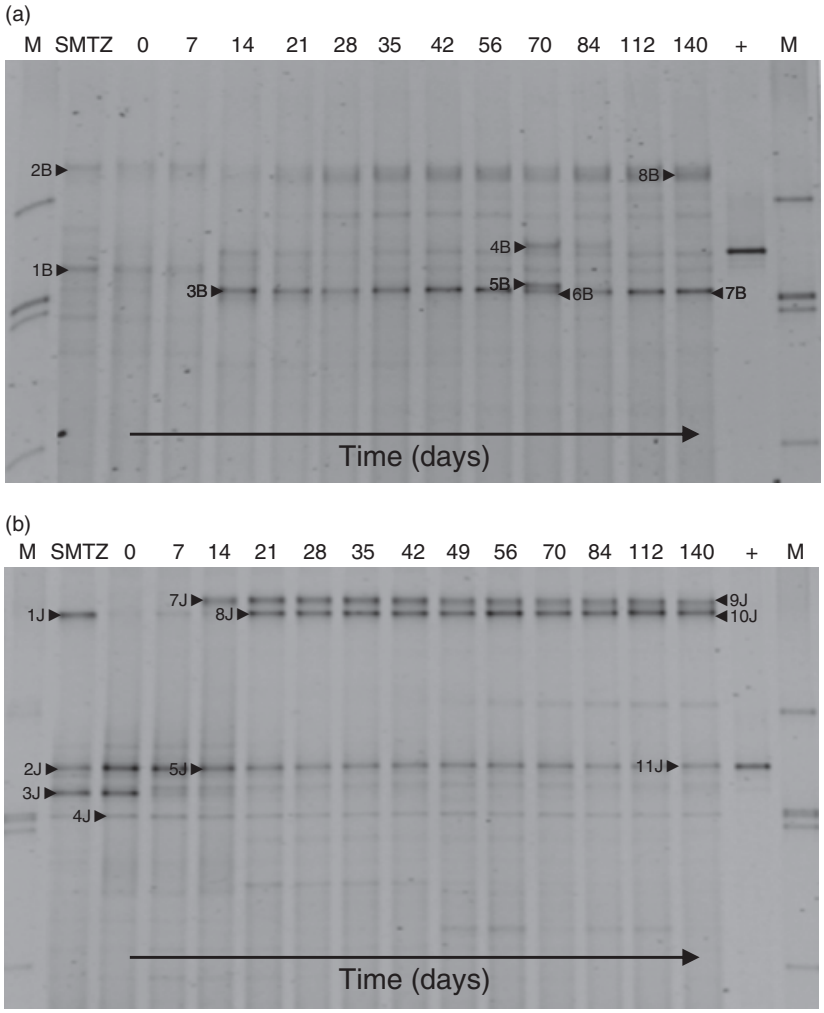

Fig. 4. (a) Bacterial and (b) JS1-targeted 16S rRNA gene PCR-DGGE analysis of Aarhus Bay SMTZ sediment slurry enrichment incubated for up to 6 months. Lane numbers represent the sample time points in days; Lanes marked SMTZ, Aarhus Bay SMTZ sediment; Lanes marked M, DGGE marker (Webster *et al.*, 2003). Numbered bands were excised and sequenced (Table 1).

bacterial 16S rRNA gene library was dominated (taxa $>20\%$) by *Gammaproteobacteria*, *Epsilonproteobacteria* and *Chloroflexi* (Fig. 2a), while after 6 months of enrichment, the bacterial population was dominated by *Deltaproteobacteria* and *Firmicutes* phylotypes, with a significant number of *Gammaproteobacteria*, *Bacteroidetes* and *Chloroflexi* sequences, as well as JS1, *Synergistetes* and TM6 (Fig. 2a).

In contrast to *Bacteria*, the dominant *Archaea* community structure, monitored by PCR-DGGE, showed no changes throughout the incubation, remaining similar to the original SMTZ sediment (Fig. 5a). All excised DGGE band sequences belonged to uncultivated members of the MBG-D/*Thermoplasmatales* group (Table 1) and were 93–97% similar to the sequences from methane seeps, subsurface marine sediments, hydrothermal vent fluids and salt marsh sediments (e.g. Huber *et al.*, 2002; Newberry *et al.*, 2004; Beal *et al.*, 2009; Nelson *et al.*, 2009). However, PCR-DGGE analysis of specific methanogen *mcrA* genes showed clear changes in the methanogenic *Archaea* population (Fig. 5b). For example, from 28 days, *Methanosarcina* sp. *mcrA* genes could be detected (Fig. 5b; Table 1), whereas, before this, only putative methane-oxidizing *Archaea* ANME-1 (*mcrA* group a; Hallam *et al.*, 2003) sequences were identified. The presence of *Methanosarcina*-related *mcrA* sequences also coincided with the detection of methane and the start of acetate consumption (Fig. 3).

PCR cloning of archaeal 16S rRNA genes (Fig. 2b) also demonstrated that the SMTZ sediment archaeal population contained ANME-1 and the MBG-D phylotypes, as well as members of the MCG and other novel *Euryarchaeota*. Throughout 6 months of incubation, all of the archaeal groups were maintained within the SMTZ sediment slurry (Fig. 2b), with the exception of the novel *Euryarchaeaota*, and in addition, *Crenarchaeota* groups MBG-B and MBG-C (Vetriani *et al.*, 1999) were also present. However, the numbers of ANME-1 sequences increased from around 7% in the SMTZ sediment to 32% of sequences after 6 months of enrichment, demonstrating that anaerobic methanotrophs were stimulated in the sediment enrichment (Fig. 2b).

Table 1. Prokaryotic 16S rRNA and *mcrA* gene sequence matches to excised DGGE bands from the Aarhus Bay SMTZ sediment slurry enrichment incubated for up to 6 months

PCR-DGGE analysis	DGGE band	Nearest match by BLASTN search (accession number)	% Sequence similarity	Phylogenetic affiliation	Isolation environment of the nearest sequence match
Bacteria 16S rRNA gene (357FGC-518R)	1B	Uncultured bacterium clone JT75-307 (AB189375)	89	*Epsilonproteobacteria*	Cold-seep sediment, Japan Trench
	2B	*Desulfovibrio* sp. midref-38 (DQ522113)	95	*Deltaproteobacteria*	Marine sediment, Oahu Island, Hawaii
	3B, 6B, 7B	*Vibrio cyclitrophicus* strain Do-49 (AB257335)	95–100	*Gammaproteobacteria*	
	4B	Uncultured bacterium clone PI_4c12d (AY580418)	93	*Epsilonproteobacteria*	Bacterioplankton, Plum Island Sound Estuary
	5B	*Marinobacter* sp. MH125a (EU052743)	96	*Gammaproteobacteria*	
	8B	DGGE band STB-726E (EU330901)	89	*Deltaproteobacteria*	Injection water from an oil reservoir, North Sea
JS1-targeted 16S rRNA gene (63F-665R and 357FGC-518R)	1J, 8J, 10J	*Thiomicrospira* sp. Tms-MPN/ 30–32 mm depth (AJ011074)	94–95	*Gammaproteobacteria*	Tidal sediment MPN culture, Wadden Sea
	2J, 4J, 5J, 11J	Uncultured bacterium clone bOHTK-29 (FJ873258)	95–100	JS1	Cold-seep sediment, Okhotsk Sea
	3J	Uncultured bacterium clone KZNMV-25-B48 (FJ712584)	96	*Gammaproteobacteria*	Gas hydrate sediment, Kazan Mud Volcano
	7J, 9J	*Desulfovibrio* sp. midref-38 (DQ522113)	100	*Deltaproteobacteria*	Marine sediment, Oahu Island, Hawaii
Archaea 16S rRNA gene (SAF-Parch519R)	1A, 2A, 3A, 5A, 6A, 7A, 8A	Uncultured archaeon clone SAT_3C10 (FJ655660)	93–97	MBG-D/ *Thermoplasmatales*	Salt Marsh sediment, Long Island Sound Estuary
	4A	Uncultured archaeon clone aOHTK-41 (FJ873204)	94	MBG-D/ *Thermoplasmatales*	Cold-seep sediment, Okhotsk Sea
	9A	Uncultured archaeon clone 33-P73A98 (AF355815)	96	MBG-D/ *Thermoplasmatales*	Hydrothermal vent fluid, Juan de Fuca Ridge
	10A	Uncultured archaeon clone NANK-A120 (AY436522)	95	MBG-D/ *Thermoplasmatales*	Subsurface sediment, Nankai Trough
Methanogen *mcrA* gene (MLF-MLR)	1M, 2M, 3M, 4M, 5M	Uncultured archaeon clone 139mcrG04 (FJ456014)	94–98	ANME1 (*mcrA* group a)	SMTZ sediment, Santa Barbara Basin
	6M, 7M, 8M	*Methanosarcina* sp. strain WH-1 (U22249)	89	*Methanosarcinales*	

See Figs 4 and 5 for DGGE gel images.

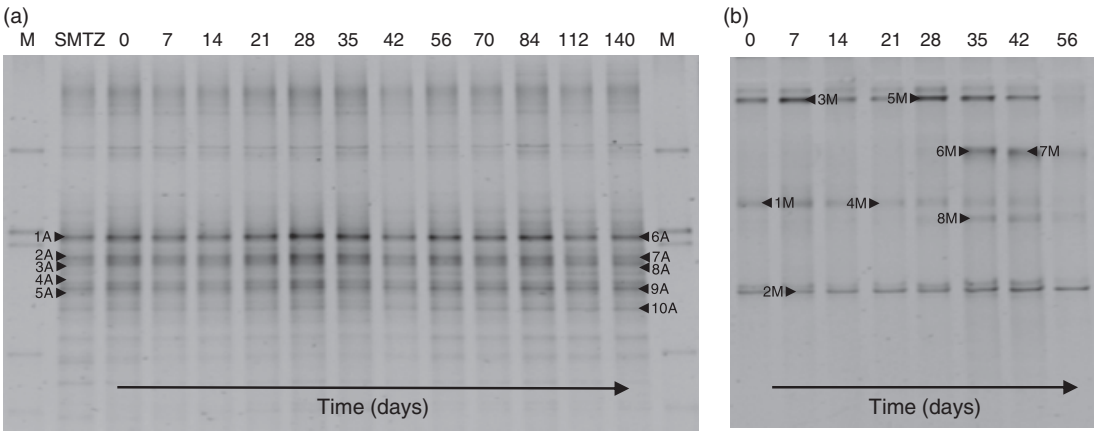

Fig. 5. (a) Archaeal 16S rRNA and (b) *mcrA* gene PCR-DGGE analysis of Aarhus Bay SMTZ sediment slurry enrichment incubated for up to 6 months. Lane numbers represent the sample time points in days; lanes marked SMTZ, Aarhus Bay SMTZ sediment; lanes marked M, DGGE marker (Webster *et al*., 2003). Numbered bands were excised and sequenced (Table 1).

Selective cultivation of SMTZ prokaryotes in 96-deep-well plates

As many of the major prokaryotic groups found in the SMTZ (eight of the 11 major bacterial and archaeal phyla) were still present in the SMTZ sediment slurry after 6 months of enrichment, this slurry was used for selective enrichment under a range of more specific conditions (Tables S1 and S2). After a further 6 months of incubation (i.e. two 3-month subcultures), the cell numbers ranged from 1.2×10^8 to 9.8×10^9 for medium 1 (Shlimon *et al.*, 2004) and 8.0×10^6 to 3.2×10^9 for medium 2 (Köpke *et al.*, 2005). Despite the cell numbers being lower in medium 2 than in medium 1, no clear patterns or trends were observed with respect to different substrate conditions in the different media, although the presence of both 10 mM acetate and 10 mM glucose consistently gave rise to the highest cell numbers in both media.

Archaeal 16S rRNA gene PCR-DGGE analysis of the 96-deep-well plates revealed that the diversity of dominant archaeal phyla after selective enrichment was considerably reduced, with only 56% and 84% of wells in plates with medium 1 and medium 2, respectively, containing *Archaea*. Sequencing excised DGGE bands showed that all *Archaea* identified in wells belonged to the *Euryarchaeota* orders *Methanosarcinales* and *Methanomicrobiales* (data not shown).

In contrast, bacterial 16S rRNA gene PCR-DGGE analysis revealed that each 96-deep-well plate had a large bacterial diversity, with medium 1 having 34 and medium 2 having 27 phylotypes (a total of 43 different phylotypes). These phylotypes represented several bacterial phyla/subphyla (*Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Beta-*, *Gamma-*, *Deltaproteobacteria*, JS1, *Synergistetes* and *Tenericutes*), and the most diverse group was the *Firmicutes*, with 23 unique phylotypes. Several (four out of eight taxa) of these cultivated bacterial phylogenetic groups were also present in the Aarhus Bay SMTZ sediment (*Chloroflexi*, *Gamma-*, *Deltaproteobacteria* and JS1) and many of the groups (seven out of nine taxa) were present in the SMTZ sediment slurry (Fig. 2; Table 2), demonstrating that this culturable diversity was still representative of the prokaryotic diversity found in the SMTZ sediment. PCR-positive wells had between one and seven bacterial phylotypes, with some wells having up to five different phylogenetic groups. Interestingly, only 13 of the 43 phylotypes identified were related ($>$ 97% sequence similarity) to previously known cultivated species, and these all belonged to phyla (*Gamma-*, *Deltaproteobacteria* and *Firmicutes*) commonly cultivated from marine sediments (Fry *et al.*, 2008). Hence, 70% of the culturable bacterial diversity in this study represented novel taxa.

The dominant bacterial phylotypes in both deep-well plates were similar, with C1 (*Chloroflexi* subphylum I), F1, F2, F3, F8 (*Firmicutes*), G2, G3 (*Gammaproteobacteria*) and S1 (*Synergistetes*) sequences, and these phylotypes were present in many plate wells, growing under a wide range of conditions, including the no-substrate controls (Table 2). Bacterial phylotypes in the no-substrate controls were presumably growing on low concentrations of substrates carried over from the SMTZ sediment slurry inoculum and/or substrates (CO_2 and H_2) produced from the Anaerocult A during incubation (Heizmann & Werner, 1989). However, each plate also had enrichment of unique phylotypes (16 phylotypes for medium 1; nine phylotypes for medium 2), which were only present infrequently and generally under more specific substrate conditions. For example, the JS1 phylotype J2 was detected in medium 1 with sulphate, but only when acetate was present, whereas *Bacteroidetes* phylotype BA1, also only present in medium 1, was only enriched with 10 mM glucose (Table 2; Tables S1 and S2).

To aid interpretation of the 96-deep-well plate results, PCA was used to explore the links between bacterial phylotypes and substrates. PCA of abundant phylotype scores from both plates yielded reasonable summaries of the data, as 43% and 46% of the variability was explained by the first three PCA components in media 1 and 2, respectively. Component plots for media 1 and 2 analysed individually (Fig. 6a and b) show some clear associations between phylotype presence and carbon substrates. For example, on both plots, glucose groups with *Gammaproteobacteria* G3 and *Synergistetes* S1 and acetate groups with *Firmicutes* F8. Additionally, acetate groups with the JS1 phylotype J2 in medium 1 and with the *Firmicutes* F12 in medium 2 (Fig. 6a and b), and glucose is associated with *Firmicutes* F11 and F15 in medium 2 (Fig. 6b). Furthermore, some of these observed PCA relationships were also further supported when the phylotype scores from the two plates were combined and analysed (Fig. 6c). Similar to the individual analysis, 46% of the variability was explained by the first three PCA components and the combined plate PCA showed that glucose grouped with S1 and G3 and acetate grouped with J2 and F8.

The correlations between the phylotype scores used in the PCA and concentration of substrate in each plate were also investigated. The results of this analysis confirmed that all groupings suggested by PCA (Fig. 6) were significantly correlated. For example, glucose correlated with phylotypes S1 ($P < 0.002$, $P < 0.01$, $P < 0.002$) and G3 ($P < 0.002$, $P < 0.05$, $P < 0.02$) and acetate correlated with J2 ($P < 0.01$, no data, $P < 0.01$) and F8 ($P < 0.01$, $P < 0.02$, $P < 0.01$) in medium 1, medium 2 and both combined, respectively. Interestingly, when the scores for all JS1 phylotypes (J1 and J2) were combined, the presence of JS1 significantly correlated with acetate ($P < 0.01$) and sulphate ($P < 0.05$). This was also the case for the combined scores for the *Deltaproteobacteria* phylotype D3, which also

Table 2. Summary of the culturable bacterial diversity and substrate range in 96-well plates inoculated with Aarhus Bay SMTZ sediment slurry enrichment as assessed by 16S rRNA gene PCR-DGGE

				Substrate conditions (mM)							
				Medium 1				Medium 2			
Phylogenetic group	Phylotype code	Nearest 16S rRNA gene sequence match in NCBI database (accession number)	% Similarity	Acetate	Glucose	Sulphate	Control	Acetate	Glucose	Sulphate	Control
Bacteroidetes	BA1	Extinct smoker bacterium Ko310 (AF550584)	91	0	10	0		–	–	–	
Chloroflexi	C1	Marine sediment enrichment clone A6 (AY540497)	93–99	0–1, 10	0–1, 10	0–5	+	0–1, 10	0–1, 10	0–5	+
Firmicutes	F1	Lake sediment clone XKE-027 (EF490125)	97–100	0–1, 10	0–1	0–5	+	0–1, 10	0–1	0–5	+
	F2	Lake sediment clone XKE-011 (EF490109)	96–100	0–1, 10	0–1	0–5	+	0–1, 10	0–1, 10	0–5	+
	F3	Niigata oil well clone Niigata-24 (AB243992)	90–94	0–1, 10	0, 1, 10	0–5	+	0–1, 10	0–1, 10	0–5	+
	F4	*Clostridium mesophilum* strain SW408 (EU037903)	95–100	10	10	0–2		1, 10	1, 10	0–2	+
	F5	*Clostridium disporicum* strain NML 05A027 (DQ855943)	98–100	10	10	0–2		1, 10	1, 10	0–2	+
	F6	Sapropel bacterium J151 (AJ630153)	98	10	10	1		10	10	1	
	F7	Tidal sediment slurry clone 13C16S1-33 (AM118010)	100	10	10	0.5		10	10	0.5	
	F8	Mesophilic methane fermentation reactor clone 30BF17 (AB330617)	92–100	0.1–1, 10	0.1–1	0–5	+	0.1–1, 10	0.1–1	0–5	+
	F9	Effluent treatment plant clone M46 (DQ640953)	91	–	–	–		0.1–0.5	0.1–0.5	0.5, 5	
	F10	Marine sediment clone MFC-9 (EU194837)	95	10	0	5	+	1	0	5	
	F11	*Clostridium perfringens* isolate SW9 (AM889037)	97	–	–	–		0.1	10	0.05, 1, 5	
	F12	*Desulfotomaculum thermoacetoxidans* (Y11573)	95	–	–	–		10	0.1	0, 1	
	F13	Subsurface groundwater clone (AB237715) 88%	88	–	–	–		1	10	5	
	F14	Uranium-contaminated aquifer clone 1013-28-CG46 (AY532583)	94	0.1	10	0.05		1	10	0.1–1, 5	
	F15	Hydrocarbon-contaminated sediment clone SRB6c31 (DQ176628)	86	–	–	–		1	10	0, 1	
	F16	Groundwater clone YWB40 (AB294309)	97	0.1	0.1	1		–	–	–	
	F17	Mesophilic biogas plant clone 182_BE3_40 (FJ825488)	91	0.1–0.5	0.1–0.5	0.5, 2	+	–	–	–	
	F18	Subseafloor clone 33-FL71B00 (AF469408)	96	0	10	0		–	–	–	
	F19	*Clostridium grantii* strain A1(X75272)	98	0.1	10	1		–	–	–	
	F20	Mesophilic biogas digester clone BL34 (EU586239)	89	10	0.1	0.05		–	–	–	
	F21	Rice paddy soil clone TSNIR001_C23 (AB487032)	85–89	1	10	1, 5		–	–	–	
	F22	*Clostridium alkalicellum* strain Z-7026 (AY959944)	97	1	10	0.5–1, 5		–	–	–	
	F23	Mesophilic biogas digester clone BS11 (EU358686)	89	10	1	5	+	–	–	–	
Betaproteobacteria	B1	Tidal flat clone BS1-0-10 (AY254969)	90	–	–	–		0	1	0.5, 5	
Gammaproteobacteria	G1	*Marinobacter alkaliphilus* strain W082 (EF114181)	100	1	1	5		1	1	0.05–1, 5	
	G2	*Marinobacterium litorale* strain IMCC1877 (DQ917760)	91–98	0–1	0.1–1	0–5	+	0–1, 10	0–1, 10	0–5	+
	G3	*Marinobacter* sp. MH125a (EF140755)	93–97	0–1, 10	0.1–10	0–5	+	0–1, 10	1, 10	0–5	+
	G4	Uranium waste pile clone GR-Sh1-128 (AJ296567)	95	–	–	–		0.1–1, 10	0–0.5	0–5	+
	G5	Alkali lake bacterium SVAL2.11 (EF522901)	89	–	–	–		10	0.1	0–0.5	
	G6	Marine sediment clone AR32 (FJ656473)	89	1	1	0.05		–	–	–	
	G7	*Marinobacterium* sp. KW44 (FJ716699)	100	10	0.1	0.5		–	–	–	
Deltaproteobacteria	D1	Peru Margin clone ODP1227B10.23 (AB177043)	97	–	–	–		1	1	0–0.05	
	D2	*Desulfuromusa succinoxidans* strain DSM 8270 (X79415)	91–98	0.1–0.5	0.1–0.5	5	+	0.5	0.5	5	
	D3	*Desulfovibrio* sp. midref-38 (DQ522113)	99	0.1–1, 10	0.1	0.1–5		10	1	0.05–5	
	D4	Tidal flat sediment clone TfP20L17 (EU362256)	94–95	0.5–1	0.5	0	+	–	–	–	
	D5	*Algidimarina propionica* strain AK-P (AY851291)	98	1	1	0–0.05		–	–	–	
	D6	Sediment clone 71 T9d-oil (FM242334)	91	10	0.1	0		–	–	–	
JS1	J1	Passaic River sediment clone PR_OTU-02 (EF165517)	96–97	10	1	0.05–5		0.5	0.5	5	
	J2	Sulphidic spring sediment clone G135 (DQ521095)	92–96	0.1–1, 10	0–1	0.05–5		–	–	–	
Synergistetes	S1	Hypersaline industrial wastewater clone RH.208-49-22 (DQ662508)	93–97	0–1, 10	1, 10	0–5	+	0–1, 10	0.5–1, 10	0–5	+
Tenericutes	T1	*Haloplasma contractile* strain SSD-17B (EF999972)	87	1	10	1		–	–	–	

+, Presence of bacterial phylotype in control wells with no substrate addition. –, Absence of bacterial phylotype in wells with substrate addition.
Data summarized from that shown in Tables S1 and S2.

correlated with acetate ($P < 0.002$) and sulphate ($P < 0.05$). In addition, phylotypes F4 and F5 occurred mainly in wells with 10 mM glucose and 10 mM acetate.

Discussion

SMTZ prokaryotic populations

Recent studies by Hamden *et al.* (2008) and Harrison *et al.* (2009) have suggested that the prokaryotic communities that are often present in the sediments, at or near the SMTZ, have a characteristic phylogenetic signature. For example, SMTZ prokaryotic populations are not only enriched in AOM-associated ANME and members of the *Deltaproteobacteria*, they also have a major component of uncultivated members of other novel groups (*Planctomycetes*, JS1, *Actinobacteria*, *Crenarchaeota* and *Thermoplasmatales*-related *Euryarchaeota*) not known to be linked with AOM (Harrison *et al.*, 2009). This also seems to be the case in the SMTZ of Aarhus Bay sediments investigated in the present study (Fig. 2; Table S3), with *Gamma-*, *Delta-*, *Epsilonproteobacteria*, JS1, *Planctomycetes*, *Chloroflexi*, MCG, ANME-1 and the MBG-D/*Thermoplasmatales* groups (Fig. 2; Table S3) all being present.

Aarhus Bay SMTZ sediment ANME-1 sequences were closely related (98% sequence similarity) to the Eel River Basin clone Ba1b1 (Hinrichs *et al.*, 1999) and the *Deltaproteobacteria* phylotypes were affiliated with the putative SRB Eel-1 group, first described in the Eel River Basin (Orphan *et al.*, 2001). Previously, ANME-1 sequences have been reported to be the dominant ANME type in the SMTZ of Aarhus Bay (Thomsen *et al.*, 2001; Aquilina *et al.*, 2010), as well as the SMTZ in nearby sediments of the Tommeliten seep area (Niemann *et al.*, 2005; Wegener *et al.*, 2008). In addition, both ANME-1 and Eel-1 group phylotypes were abundant in the SMTZ of Santa Barbara Basin sediments (Harrison *et al.*, 2009). A comparison of other methane-associated sediments where members of both groups have been found indicates a possible relationship between Eel-1 *Deltaproteobacteria* and ANME-1 (Harrison *et al.*, 2009), and their dual presence in many SMTZ sediments may suggest that they are physiologically adapted to this type of environment.

In diffusive marine sediments, the distribution of AOM is restricted to the SMTZ and the AOM rates are often lower (10 nmol cm^{-3} day^{-1}) than those at cold seeps (Knittel & Boetius, 2009). This is the case in this and other studies of Aarhus Bay sediments (Fig. 1; Dale *et al.*, 2008; Aquilina *et al.*, 2010), as well as in the nearby sediments of the Skagerrak (Parkes *et al.*, 2007) and North Sea Tommeliten site (Niemann *et al.*, 2005). However, despite such low rates, AOM is still an effective barrier to prevent methane escaping to the water column in Aarhus Bay (Dale *et al.*, 2009) and other diffusively controlled sediments (e.g. Skagerrak, Parkes *et al.*, 2007). Similarly, the population densities of ANME in diffusive sediments are restricted to the SMTZ and are also low ($<10^6$ cells cm^{-3}; Knittel & Boetius, 2009), although the overall total prokaryotic cell numbers are generally enhanced at SMTZs (Fig. 1b; Parkes *et al.*, 2005). This may suggest that many other prokaryotes are active and

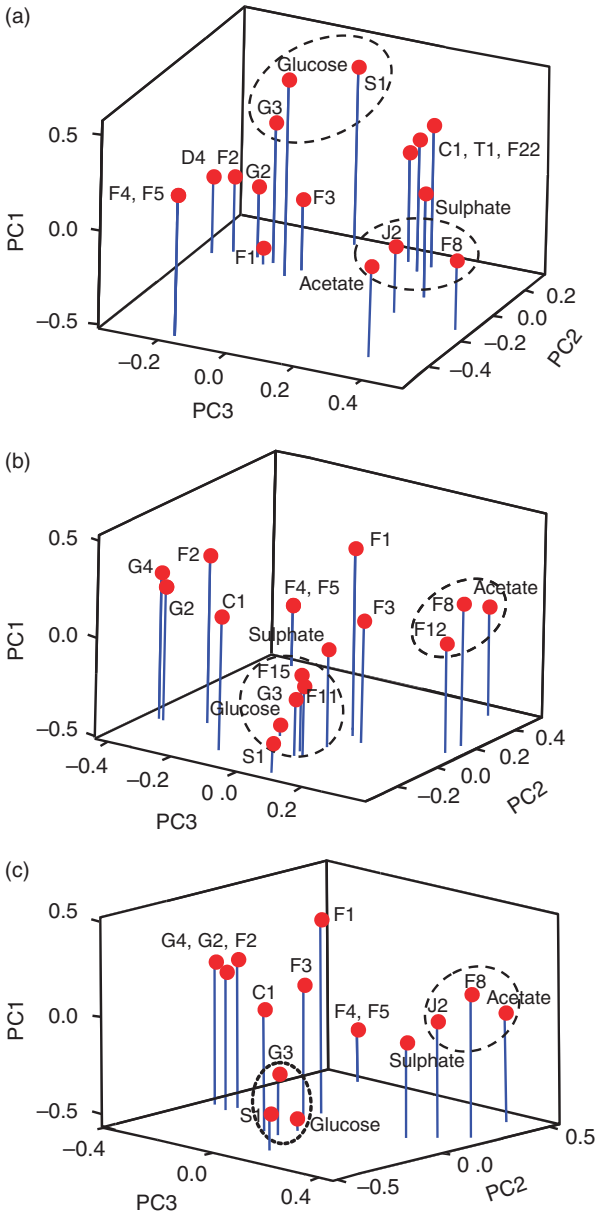

Fig. 6. Three-dimensional plots of the loadings for the first three principal components (PC1–PC3) from the analysis of abundant bacterial phylotypes in 96-deep-well plates inoculated with Aarhus Bay SMTZ sediment slurry. PCA of plates containing (a) medium 1, (b) medium 2 and (c) both media combined. Points are representative of phylotype assignations and substrates analysed. Dotted lines represent the PCA relationships discussed in the text.

adapted to the conditions of this unique biogeochemical zone, with substrates other than methane being important. Such physiological adaptation may also be an important factor for members of the JS1, *Planctomycetes* and *Chloroflexi* (Fig. 2; Table S3), all of which are found in Aarhus Bay SMTZ sediments and other organic-rich or methane-associated sediments (e.g. Inagaki *et al.*, 2006; Webster *et al.*, 2006a, 2007; Blazejak & Schippers, 2010). However, because the majority of sequences belonging to these groups are not related to cultivated bacteria, their metabolic and physiological characteristics remain unknown; this highlights the importance of developing cultivation methods to enrich and isolate them.

Enrichment of SMTZ bacteria

Sediment slurries have been used successfully to enrich anaerobic bacteria from a number of environments (e.g. Bedard *et al.*, 2006; Oren, 2006; Nauhaus *et al.*, 2007) and the slurry conditions used in this study have been used to maintain and study heterotrophic prokaryotic functional diversity in marine sediments (Webster *et al.*, 2006b, 2010). Because the activity and growth of prokaryotes in the Aarhus Bay SMTZ ultimately depends on organic matter, with possible utilization of other substrates in addition to methane, we used a similar approach to enrich SMTZ prokaryotes, with the additional condition of maintaining the low sulphate concentration that is characteristic of the SMTZ. This approach was successful, with representative SMTZ prokaryotes being enriched, which provided an effective inoculum for further selective cultivation of SMTZ prokaryotes. However, this further enrichment using a range of substrates and growth conditions resulted in a loss of SMTZ archaeal diversity and conditions were clearly more selective for SMTZ bacteria. PCR-DGGE analysis showed that only methanogenic *Archaea*, belonging to the previously cultivated orders *Methanomicrobiales* and *Methanosarcinales*, were able to compete with *Bacteria* under these conditions. *Methanomicrobiales* can use H_2/CO_2 produced from the Anaerocult A during incubation (Heizmann & Werner, 1989), and the *Methanosarcinales* are metabolically diverse and utilize a wide range of substrates including the added acetate and H_2/CO_2 (Liu & Whitman, 2008).

In contrast to the limited cultivation of *Archaea*, our approach resulted in the cultivation of a diverse population of novel SMTZ *Bacteria*, with different bacterial groups being enriched by specific substrate combinations, as demonstrated by PCA (Fig. 6). Cultivation in the presence of acetate selected for JS1 and some *Firmicutes* (F8 and F12), whereas glucose selected for *Synergistetes* and phylotypes belonging to the *Gammaproteobacteria* (G3) and *Firmicutes* (F11 and F15). On the other hand, some *Bacteria* such as *Chloroflexi*, other *Gammaproteobacteria* and *Firmicutes* phylotypes, were selected for over a range of conditions with acetate and glucose (Tables S1 and S2). This difference in cultivation for JS1 and *Chloroflexi* reinforces environmental data suggesting that members of these two phyla can occupy different sedimentary habitats (Webster *et al.*, 2007).

Chloroflexi and JS1 are two phylogenetic groups often found to dominate in various marine subsurface sediments (Fry *et al.*, 2008), as well as being present regularly in and around the SMTZ (Harrison *et al.*, 2009), but their metabolism in these environments is unknown. The results of this study, showing that JS1 and *Chloroflexi* bacteria can be cultivated in sediment enrichments on acetate or glucose, support previous findings that some members of these groups of bacteria are heterotrophic (Webster *et al.*, 2006b; Yamada & Sekiguchi, 2009). In addition, in wells with a positive growth of JS1, all added sulphate was depleted and acetate concentrations were 1–40% of the original concentration (data not shown). Stable-isotope probing in anoxic sediment slurries with low sulphate has already suggested that some members of JS1 are able to utilize acetate and glucose or glucose metabolites (Webster *et al.*, 2006b), and our results further support this in mixed culture and after subculture under more specific conditions (Table 2). Whereas cultivated members of the *Chloroflexi* subphylum I have only previously been shown to degrade carbohydrates and amino acids under anaerobic conditions (Yamada & Sekiguchi, 2009), our results demonstrate acetate utilization by members of subphylum I under several mixed culture conditions (Table 2).

Cultivated members of the *Synergistetes* are obligate anaerobes often associated with animal microbial communities or as opportunistic pathogens, although sequences have been identified in a number of anaerobic environments, such as anaerobic digestors and petroleum reservoirs (Godon *et al.*, 2005; Pham *et al.*, 2009). However, the metabolism and role of *Synergistetes* in anaerobic ecosystems remains unknown. *Synergistetes* seem to be anaerobic amino-acid degraders (Godon *et al.*, 2005) and only a few members of this group, such as *Thermanaerovibrio velox* (Zavarzina *et al.*, 2000) and *Anaerobaculum* species (Rees *et al.*, 1997; Menes & Muxi, 2002), are also able to use carbohydrates. The *Synergistetes* phylotype S1 in our experiment was often the dominant bacterium in wells with high glucose concentrations (10 mM glucose), occurring in mixed culture with and without sulphate (Tables S1 and S2), which suggests a fermentative metabolism.

Implications for enriching SMTZ prokaryotic communities

The characterization and isolation of prokaryotic groups associated with the unique physicochemical and ecological environment within SMTZ sediments is essential for our understanding of the microbial ecology of this important

geochemical interface, as many more prokaryotes are associated with this zone other than known ANMEs and their syntrophs. In this study, the use of a heterotrophic sediment slurry enrichment plus further selective enrichment under a series of specific conditions proved to be a useful technique to obtain important metabolic/physiological information about novel *Bacteria* associated with SMTZs, such as members of JS1 and *Chloroflexi*. This strategy could lead to the potential isolation of SMTZ prokaryotes. In addition, such an approach should provide samples sufficiently enriched in novel prokaryotes to facilitate metagenomic analysis of uncultivated prokaryotes.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. *Bacteria* identified in a 96-deep-well plate inoculated with Aarhus Bay SMTZ sediment enrichment containing medium 1 with varying concentrations of acetate, glucose and sulphate.

Table S2. *Bacteria* identified in a 96-deep-well plate inoculated with Aarhus Bay SMTZ sediment enrichment containing medium 2 with varying concentrations of acetate, glucose and sulphate.

Table S3. Prokaryotic 16S rRNA gene sequence matches to clone sequences from Aarhus Bay SMTZ sediment and the SMTZ sediment slurry enrichment incubated for 6 months.

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