Identification of toluene degraders in a methanogenic enrichment culture

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Abstract

Methanogenic biodegradation involves the cooperative metabolism of syntrophic bacteria that catalyse the initial attack and subsequent degradation of hydrocarbons, and methanogens that convert intermediates such as hydrogen and carbon dioxide, formate, and/or acetate to methane. The identity of syntrophic microbes and the nature of their interactions with other syntrophs and methanogens are not well understood. Furthermore, it is difficult to isolate the organisms responsible for the initial activation and subsequent degradation of hydrocarbon substrates under methanogenic conditions due to the thermodynamic relationships that exist among microbes in methanogenic communities. We used time-resolved RNA stable isotope probing and RT-qPCR to identify the organisms involved in the initial attack on toluene and subsequent degradation reactions in a highly enriched toluene-degrading methanogenic culture. Our results reveal the importance of a Desulfo sporinus sp. in anaerobic toluene activation in the culture. Other organisms that appear to play roles in toluene degradation include Syntrophaceae, Desulfovibrionales and Chloroflexi. The high bacterial diversity observed in this culture and the extensive labelling of different phylogenetic groups over the course of the stable isotope probing experiment highlight the complexity of the relationships that exist in methanogenic ecosystems.

Introduction

Hydrocarbons are found naturally in the deep subsurface; however, widespread contamination of shallow subsurface environments with hydrocarbons has led to regulatory concerns due to negative impacts on human and ecosystem health. Monoaromatic hydrocarbons such as BTEX (benzene, toluene, ethylbenzene and xylenes) are of particular concern due to their toxicity and relatively high solubility in water (Dean, 1985). While these compounds can be readily degraded aerobically, hydrocarbon spills into the subsurface result in anoxic conditions. Thus, in such environments, hydrocarbon degradation occurs anaerobically, a process that is less well understood. To facilitate the development of in situ bioremediation technologies, a detailed understanding of the processes and the organisms involved in anaerobic hydrocarbon metabolism is imperative.

Toluene is a model compound for studying anaerobic hydrocarbon degradation and can be oxidized anaerobically coupled to the reduction of sulphate, iron(III), manganese, nitrate and chloride anoxones, as well as under anaerogenic phototrophic and methanogenic conditions (Widdel et al., 2006; Heider, 2007). Several isolates have been obtained that are capable of anaerobic toluene degradation; however, no bacteria involved in methanogenic toluene metabolism have been isolated (Cupples, 2011). In environments where metabolism is governed by syntrophic processes it can be difficult to isolate the organisms responsible for the activation and subsequent degradation of a substrate. This is often the case in methanogenic environments where individual organisms can rarely be purified due to the thermodynamically interdependent relationships between the syntrophic bacteria that catalyse the initial attack and subsequent degradation of a substrate and the methanogens that produce the end product, methane. Desulfitubilillum alkenivorans AK-01 is the single isolate known to methanogenically degrade a hydrocarbon substrate.
(hexadecane) in co-culture with a methanogen (Callaghan et al., 2012).

Previously, we described a methanogenic toluene-degrading culture derived from a gas-condensate-contaminated aquifer (Fowler et al., 2012). This culture completely mineralizes toluene to methane using fumarate addition as the hydrocarbon activating mechanism. The consortium contains a diverse community consisting primarily of Firmicutes, Chloroflexi, Spirochaetes, Deltaproteobacteria and acetoclastic and hydrogenotrophic methanogens as determined using 16S rRNA gene pyrotag sequencing (Fowler et al., 2012). These results are in line with a previous study that found the presence of Firmicutes, Deltaproteobacteria and acetoclastic and hydrogenotrophic methanogens in a different methanogenic toluene degrading enrichment culture (Ficker et al., 1999). The organism(s) carrying out the initial attack on toluene was not identified in either study, although Fowler et al. (2012) hypothesized, based on sequence abundance, that a member of the Clostridiales was a key toluene degrader. Recent DNA-stable isotope probing (SIP) studies carried out on methanogenic hydrocarbon degrading cultures have implicated members of the Syntrophaceae (benzene and hexadecane) and Peptococcaceae (toluene) in hydrocarbon activation (Sakai et al., 2009; Cheng et al., 2013; Sun et al., 2014). In this study, we employed time-resolved RNA-SIP using 13C7-toluene in order to follow the flow of isotope label through the culture. Specifically, we focused on identifying the bacteria that carry out the initial attack and subsequent degradation of toluene. The activity of the key bacteria identified using RNA-SIP analysis was then examined using RT-qPCR to confirm their roles in methanogenic toluene metabolism in this culture.

Materials and methods

Culture incubations

A sediment-free toluene-degrading methanogenic culture originally enriched from a gas condensate-contaminated aquifer was used in this study. The culture has been maintained by routine transfer and substrate amendment with toluene as the sole electron donor under methanogenic conditions for over 10 years and was repeatedly shown to mineralize toluene (c. 20 µmol/50 mL culture) to methane within c. 40 days (Fowler et al., 2012). For stable isotope probing experiments, 20-mL cultures were prepared anaerobically in 40-mL serum bottles and were sealed with butyl rubber stoppers. Cultures were incubated with 1 µL (9.4 µmol) 13C7-labelled toluene (99%, Sigma-Aldrich) or unlabelled toluene (Aldrich) and were sampled at days 1, 3 and 7. At each time point, two bottles were sacrificed for RNA extraction, one incubated with 12C7-toluene and one incubated with 13C7-toluene.

RNA extraction, density gradient centrifugation and fractionation

RNA was extracted using the Qiagen AllPrep DNA/RNA mini kit with bead beating. Pelleted cells were added to a 2 mL tube containing 0.3 g of 0.1 mm and 0.1 g of 0.5 mm zirconia/silica beads (BioSpec Products) and 400 µL of buffer RLT Plus. Cells were disrupted by bead beating for 1 min at 6.0 m s⁻¹. The extraction was then carried out according to the manufacturer’s protocol. To separate 12C-RNA and 13C-RNA, 129 ng of RNA was added to 2.8 mL of caesium trifluoroacetate (GE Healthcare), and 119 µL of formamide (Sigma) to a total volume of 3.5 mL. Density gradients were formed by centrifugation at 150 000 g (20 °C) for 44 hours as described (Zemb et al., 2012). Gradients were fractionated into 16 × 200 µL fractions by displacement at 0.2 mL min⁻¹. The density of fractions was determined by weighing 100 µL aliquots (Zemb et al., 2012).

RNA precipitation, quantification, reverse transcription and amplification

RNA was recovered from each fraction by isopropanol precipitation and was quantified using Quant-iT RiboGreen RNA reagent (Invitrogen). Fractions 1–10 from all gradients were reverse transcribed. Fractions 11–16 were not analysed further as they did not contain substantial amounts of RNA. RNA was denatured (65 °C, 5 min) with bacterial 16S rRNA gene primer 530R (0.8 µM; Table 1) then AMV reverse transcriptase (10 U, Promega), 5x Tris-HCl reaction buffer and deoxynucleotides (2 mM each) were added and tubes were incubated at 37 °C for 1 hour in a 25 µL sample volume.

PCR amplification of cDNA was carried out using bacterial 16S rRNA gene primers 530R and GC338F (5'-CGCCCGCCTGCCGCCTGGCCCTGCGGGCCCGCCC-3') to amplify the V3 region, at the following conditions: 95 °C 2 min, 30 cycles of 95 °C 30 s, 62 °C 30 s, 72 °C 1.5 min; and 72 °C 10 min.

Denaturing gradient gel electrophoresis

DGGE was carried out as described previously (Shartau et al., 2010). Briefly, 200–250 ng of cDNA was loaded onto a 6.5% (w/v) polyacrylamide gel with a 40–75% denaturing gradient of urea and formamide. Gels were run at 75 V, 60 °C for 16 h and were stained with SYBR
Green (Invitrogen). Bands were visualized and excised under UV light and incubated in 50 μL Tris-EDTA buffer overnight. Pelleted bands (18 000 g, 5 min) were used as a template for re-amplification with bacterial 16S rRNA gene primers 338F and 530R (Table 1). The program used was as above but with an annealing temperature of 55 °C. Amplicons were purified using the Qiaquick Gel Extraction Kit (Qiagen).

**Sequencing and phylogenetic analysis**

Purified amplicons were sequenced at the UCDNA laboratory on an Applied Biosystems 3730xl 96 capillary genetic analyser (University of Calgary, Canada). Chimeric sequences were screened for using MALLARD 1.02 (Ashelford et al., 2006). No chimeras were identified. Sequences were compared to the NCBI non-redundant nucleotide database using BLASTN to determine taxonomic affiliation. Sequences were submitted to GenBank and have been assigned accession numbers JX171701–JX171735.

Multiple sequence alignments of 16S rRNA sequences from DGGE bands, and 16S rRNA gene sequences from closely related cultured organisms and related organisms or clones associated with anaerobic hydrocarbon metabolism were made in T-coffee (Notredame et al., 2000). Bootstrapped maximum likelihood trees were made in PHYLIP (Felsenstein, 1993).

**Quantification of key organisms identified in RNA-SIP**

For RT-qPCR experiments, 10 mL incubations were prepared from a single culture that had been amended with 0.01% peptone (in addition to toluene) to enhance growth. Cultures that were deemed free of toluene for 14 days were incubated with 1 μL of unlabelled toluene and were sacrificed in triplicate by centrifugation at 12 000 g for 10 min for DNA and RNA extraction on days 1, 3, 8 and 20. Throughout the time course incubation, methane was monitored by gas chromatography as previously described (Fowler et al., 2012). Replicate cultures that were not exposed to toluene were similarly sacrificed. RNA was subject to DNaseI treatment (Invitrogen) and purification with the Zymoresearch Clean and Concentrate-5 kit (Zymoresearch). RNA samples were reverse transcribed using SuperscriptIII (Invitrogen) and cDNA was treated with RNase H according to the manufacturer’s protocol (Invitrogen). cDNA samples were then subject to RT-qPCR analysis using taxon-specific and bsSA gene primers and total rRNA genes were quantified using universal bacterial 16S rRNA gene primers (Table 1).
Primer selection, design and optimization and qRT-PCR

Primers targeting the genera Desulfosporosinus, Clostridium, Desulfovibrio and Syntrophus were sought in the literature and were tested and optimized using DNA from the toluene-degrading culture (Table 1). In the case that amplification with published primer sets was unsuccessful, primers were modified based on existing sequence data from the culture (Fowler et al., 2012). Due to the inability of pre-existing primer sets to efficiently amplify bssA in this culture, new primers were designed in Primer3 to target bssA using sequence data from the culture (Fowler et al., 2012). Newly designed primers were screened against the NCBI NR database to test for non-specific binding. All primer sets were optimized by performing temperature gradients using conventional PCR. To generate standards for quantification, amplicons generated using optimized primer sets were cloned using the Topo TA cloning kit (Invitrogen) and were transformed into Escherichia coli. Positive transformants were selected and grown in 5 mL of LB medium. Plasmid DNA was extracted using the Qiagen Miniprep kit and was sequenced (Eurofins, Louisville, KY). Insert sequences were compared to the NCBI NR database to ensure that the appropriate fragment had been amplified. Plasmid DNA was diluted from 10^2 to 10^9 copies μL^{-1} and was used to generate standard curves. Slopes of the standard curves ranged from -3.29 to -3.43 with efficiencies ranging from 95.7% to 99.7%.

RT-qPCR and qPCR reactions were carried out in triplicate in 10 μL reactions consisting of 500 nM of each primer, 2X EvaGreen Supermix (Bio-Rad) and 1 μL of template DNA in a C1000 thermocycler (Bio-Rad) under the following thermocycling conditions: 95 °C 3 min, 40 cycles of 95 °C 20 s, 25 s at the optimized annealing temperature (Table 1), followed by melt curve analysis. This procedure was slightly altered when the Syntrophus primers were used due to the larger amplicon size generated by these primers: 95 °C 3 min, 40 cycles of 95 °C 20 s and 57 °C 35 s followed by melt curve analysis. Melt curve analysis revealed the presence of a small primer dimer peak in no template control samples of bssA and universal bacterial primers. A single dominant peak was seen for each primer, though peaks were often slightly wider in community DNA samples than in standards, which likely reflects the presence of greater than one phylotype of each organism in community DNA. The bacterial universal primers exhibited a substantially wider peak than taxon-specific primers, likely reflecting the greater sequence diversity of these amplicons.

Results

Exposure to ^13C_7-toluene and RNA gradient ultracentrifugation

An established methanogenic toluene-degrading enrichment culture that has been routinely transferred for over 10 years was incubated with 9.4 μmol of either ^13C_7-toluene or unlabelled toluene and subject to RNA-SIP analysis in order to identify the active toluene-degrading bacteria in the culture. Earlier efforts to carry out RNA-SIP on this culture revealed that all culture members were labelled by day 11 (whereby 10–15% toluene was consumed; data not shown), and thus RNA was extracted from incubations containing ^13C_7-toluene or ^12C_7-toluene on days 1, 3 and 7 following substrate amendment and was subject to density gradient ultracentrifugation. To avoid introduction of trace oxygen to culture due to sampling for substrate analysis, toluene was not measured although previous incubations showed that 1 μmol toluene would be consumed after 7 days of incubation (Fowler et al., 2012). Fractionated gradients containing RNA from labelled or unlabelled pulses were subjected to DGGE fingerprinting (Fig. 1).

Bacterial diversity of toluene-degrading enrichment determined by DGGE

The major bands from DGGE gels were excised, re-amplified and sequenced. Sequences were then compared to the NCBI NR database in order to determine the taxonomic affiliation of the microbes represented by specific bands. Supporting Information, Table S1 provides the taxonomic identifications of all the bands excised and sequenced in this study. A diverse group of bacteria was identified in this culture, belonging mainly to the Deltaproteobacteria, Firmicutes and Spirochaetes (Fig. 2, Table S1). One band was found in BLASTN searches to be related to both the Firmicutes and Chloroflexi, and in phylogenetic analysis grouped with the Chloroflexi (band J4, Figs 1 and 2). Sequenced bands had between 82% and 99% identity to their closest cultured relatives, with most bands exhibiting <93% identity to cultured organisms over the sequenced region (Table S1).

Identification of bacteria involved in toluene degradation by RNA-SIP

In order to identify bacteria involved in toluene degradation, DGGE fingerprints of gradient fractions ranging in buoyant density from heavily labelled (1.82 g mL^{-1}) to unlabelled (1.79 g mL^{-1}) were compared within ^13C_7-toluene-incubated samples and to ^12C_7-toluene-incubated...
controls (Whiteley et al., 2007) (Fig. 1). At all time points in both labelled and unlabelled samples, Band C was found to be a dominant band, indicating that it may play a key role in the culture. In labelled samples on day 1, this band was observed to become partially labelled and became progressively more heavily labelled in day 3 and 7 samples incubated with $^{13}$C$_7$-toluene (Fig. 1). Band C represents an organism closely related to Desulfosporosinus sp. DB (96% identity) and to uncultured Desulfosporosinus spp. found in methanogenic BTEX-degrading enrichments derived from oil sands tailings (99% identity, Siddique et al., 2012) and proposed toluene degraders in a methanogenic toluene-degrading culture (98 and 97% identity, respectively, Table S1, Sun et al., 2014). In the sample incubated with $^{13}$C$_7$-toluene for 3 days, two additional bands were enriched in heavy and intermediate fractions (bands M and D) and remained enriched in heavy fractions in day 7 $^{13}$C$_7$-toluene-incubated samples. Band M was more intense in DGGE gels from $^{13}$C$_7$-toluene-incubated samples relative to $^{12}$C$_7$-toluene-incubated samples (Fig. 1). Band M represents an organism related to cultured Desulfovibrio spp. (93% identity) including those cultured from or identified in oil reservoirs (93 and 94% identity respectively; Table S1) (Grabowski et al., 2005; Mbadinga et al., 2012). Band D was more intense in day 3 and 7 $^{13}$C$_7$-toluene-incubated samples than at...

**Fig. 1.** Denaturing gradient gel electrophoresis (DGGE) community profile from density-resolved $^{13}$C$_7$- and $^{12}$C$_7$-toluene-incubated samples. (a) Day 1 $^{13}$C$_7$-toluene-incubated sample (left) and $^{12}$C$_7$-toluene-incubated sample (right). (b) Day 3 $^{13}$C$_7$-toluene-incubated sample (left) and $^{12}$C$_7$-toluene-incubated sample (right). (c) Day 7 $^{13}$C$_7$-toluene-incubated sample (left) and $^{12}$C$_7$-toluene-incubated sample (right). Numbers indicate buoyant densities of CsTFA gradient fractions with values ranging from 1.78–1.80 g mL$^{-1}$ typically representing unlabelled ($^{12}$C) RNA, and 1.81–1.83 g mL$^{-1}$ representing labelled ($^{13}$C) RNA. Arrows indicate the enrichment of specific DGGE bands in heavy fractions (please refer to text for details).
comparable buoyant densities in $^{12}$C$_7$-toluene-incubated samples (Fig. 1). Sequencing of band D revealed that this organism is related to members of Syntrophus and Smithella genera (up to 95% identity) which are commonly identified in oil-impacted environments (Table S1, Acosta-Gonzalez et al., 2013).

On day 7, two additional organisms, represented by bands H1 and J4, appeared to incorporate labelled carbon. Band H1 represents a relative of Desulfovibrio sp. and is related to the organism represented by band M, which became labelled on day 3 (Table S1). It likely plays a similar role in the culture to the organism represented by band M. An additional band, J4, appeared at a similar location to bands J1, J2 and J3 in $^{13}$C-labelled samples on day 7. While bands J1, J2 and J3 are most closely related to the genera Clostridium and Youngiibacter (formerly

Fig. 2. Maximum likelihood tree showing the affiliation of 16S rRNA fragments from DGGE bands with reference sequences from cultured relatives and select microbes associated with anaerobic hydrocarbon metabolism. Bootstrap values > 60 are shown (based on 100 data sets). The tree was rooted using the Spirochaetes clade.
Acetivibrio; up to 99% identity), band J4 is most closely related in BLASTN searches with the genus Peptostreptococcus (90% identity), but in phylogenetic analysis affiliates with Dehalococcoides sp. of the phylum Chloroflexi (Fig. 2).

Quantification of key organisms involved in methanogenic toluene degradation

To confirm the results of RNA-SIP analysis and to obtain a more quantitative view of the activity of different taxonomic groups during toluene metabolism, a 20-day time course experiment following changes in copy number of 16S rRNA of various taxa identified in the RNA-SIP analysis in response to toluene exposure was undertaken. In addition to Desulfoosporinus sp., Desulfovibrio sp., and Syntrophus sp., identified in RNA-SIP analysis, Clostridium was also targeted as it was found to be a dominant organism in previous pyrosequencing analysis (Fowler et al., 2012). Total bacterial 16S rDNA was monitored. In addition, the expression of bssA, the gene encoding the catalytic subunit of benzylsuccinate synthase involved in toluene activation by fumarate addition in this culture, was examined (Fowler et al., 2012). Quantification of total bacterial 16S rDNA in the culture revealed a small and non-significant change in cell numbers of about 0.6-fold over the course of 20 days, which indicates that changes in 16S rRNA abundance were due almost solely to changes in transcription and were not strongly impacted by cell growth (Fig. 3). During the time course experiment, 12 μmol CH₄ was produced. Based on the stoichiometric reaction of methanogenic toluene metabolism, this amount of methane would theoretically account for 2.7 μmol toluene consumption (toluene was not measured due to concern regarding the introduction of trace amounts of oxygen). However, this estimation is likely low as there is typically a lag in methane production following toluene depletion and between 10 and 25% of carbon from toluene is not converted to methane (Fowler et al., 2012).

Consistent with the results obtained from RNA-SIP, the transcription of 16S rRNA from Desulfoosporinus sp. increased significantly after 1 day of incubation with toluene. This high level of expression continued through days 3–8 and exhibited a much larger and immediate increase than all other community members tested in response to toluene exposure suggesting that this organism is most likely involved in toluene activation (Fig. 3). Surprisingly, a significant increase in bssA expression did not accompany the increase in Desulfoosporinus sp. activity, despite previous evidence that toluene is activated by fumarate addition in this culture (Fowler et al., 2012). Though a small increase in bssA expression was observed at each point relative to unamended cultures, these changes were not significant (Fig. 3). It is a possibility that though the primers used for bssA amplification here were designed based on the only identified bssA gene from this culture, this bssA gene was not involved in toluene activation by Desulfoosporinus sp.

The activities of both Syntrophaceae and Desulfovibrio sp. increased on day 8, consistent with the partial labelling of these organisms beginning on day 3 in RNA-SIP analysis (Fig. 3). In particular, Syntrophaceae activity increased gradually over time with significantly increased activity on days 8 and 20 relative to time 0 activity. While it is possible that these organisms are also involved in toluene activation, it seems more likely that they play a role in the downstream metabolism of toluene due to the delay in both labelling in RNA-SIP and in the increase in activity when exposed to toluene. Increases in Clostridiurn sp. activity were not significant, so it seems unlikely that Clostridiurn sp. plays a direct role in toluene metabolism as it was also not observed to incorporate carbon from toluene over 7 days (Fig. 3). These results contrast somewhat with previous results in which Clostridiurn sp. was found to be a dominant community member based on 16S rDNA pyrotag sequencing (Fowler et al., 2012). Though identified in the DGGE analysis conducted in this study, the prevalence of Clostridiurn sp. was low relative to other members of the culture (e.g. Desulfoosporinus sp.,...
Desulfovibrio sp.). The most likely explanation for these observations is primer bias, as a different universal primer set was used in this study. A shift in community composition since the previous study seems unlikely as this culture has been incubated under the same conditions for over 10 years. Further metagenomic sequencing of this culture may provide insight into the true abundance of Clostridium sp. in this culture.

Discussion

Nucleic acid based SIP has previously been carried out on cultures capable of anaerobic toluene metabolism under sulphate-, nitrate- and iron-reducing conditions, and most recently, for a methanogenic culture (Bombach et al., 2010; Winderl et al., 2010; Pilloni et al., 2011; Sun & Cupples, 2012; Sun et al., 2014). In this study, we identified the microorganisms that are presumably involved in the initial attack on toluene and subsequent reactions in a methanogenic enrichment culture using RNA-SIP and RT-qPCR. Methanogenic hydrocarbon degrading cultures exist at the lower energetic limits of life and have long generation times. In light of this, RNA was considered to be a more useful molecule than DNA to measure the activity of this culture due to its rapid turnover, particularly by microbes that are actively metabolizing a substrate (Whiteley et al., 2007). Using RNA for this study allowed for much shorter incubation times than would have been required for DNA analysis in this culture and provided adequate sensitivity to obtain meaningful results.

Organisms from four different bacterial phyla (Deltaproteobacteria, Firmicutes, Spirochaetes and Chloroflexi) were identified in our RNA-SIP/DGGE analysis, with members of several of these phyla incorporating labelled carbon over the course of 7 days. The high bacterial diversity found is supported by previous results in which this culture was found to contain 173 OTUs, with the most abundant bacteria belonging to the phyla Firmicutes, Chloroflexi, Spirochaetes and Deltaproteobacteria (Fowler et al., 2012). The highly enriched nature of this culture and the observation of the incorporation of label into numerous phyla within 7 days suggest that all of these organisms play a role in the transformation of toluene to methane and underlines the complex interactions that exist in methanogenic cultures and environments.

The early incorporation of $^{13}$C-label into Desulfosporosinus sp. on day 1 combined with a rapid increase in cell activity in response to toluene exposure suggests that it is most likely the organism primarily responsible for toluene activation (Figs 1 and 3), supporting our previous hypothesis that a member of the Clostridiales (the order into which Desulfosporosinus falls) was a key hydrocarbon-degrader in the toluene-degrading enrichment (Fowler et al., 2012). A number of studies in recent years have implicated members of the family Peptococcaceae (also within the Clostridiales) in BTEX metabolism under iron- and sulphate-reducing and now methanogenic conditions (Morasch et al., 2004; Kunapuli et al., 2007; Abu Laban et al., 2009; Herrmann et al., 2010; Winderl et al., 2010; Sun & Cupples, 2012; Sun et al., 2014). Given that many microbes are able to use multiple electron acceptors and/or metabolize fermentatively (coupled with the similarity in redox potential at which iron and sulphate reduction and methanogenesis occur), it is not surprising that similar organisms are involved in the activation of BTEX under diverse anaerobic conditions. The specific Desulfosporosinus sp. identified here has high 16S rRNA sequence similarity to both the Desulfosporosinus sp. recently identified to activate toluene under methanogenic conditions and to a Desulfosporosinus sp. identified in BTEX-degrading methanogenic cultures derived from oil sands tailings (98 and 99% respectively) suggesting that members of this clade may be specifically adapted to BTEX-containing environments (Siddique et al., 2012; Sun et al., 2014).

Interestingly, a substantial increase in bssA expression did not accompany the significant increase in Desulfosporosinus sp. activity on day 1 (Fig. 3). The abundance of bssA transcript was steady over the 20 day time course, and was not significantly higher compared with unamended cultures (Fig. 3). Transcription of bssA has previously been shown to be induced by the presence of toluene under methanogenic conditions (Washer & Edwards, 2007), so the reason for this is unclear, as both DNA sequence data and metabolite analysis support fumarate addition as the toluene activation mechanism in this culture, and members of this clade have previously been shown to activate monoaromatic hydrocarbons by fumarate addition (Morasch et al., 2004; Fowler et al., 2012).

Additional organisms that incorporated carbon from toluene and exhibited increased activity over the course of toluene exposure were a relative of Syntrophus/Smithella spp. (up to 95% identity) and a relative of Desulfovibrio sp. (93% identity; Figs 1 and 3).

Members of the Smithella/Syntrophus genera are typically fermentative organisms that are often found in association with methanogens and are frequently identified in hydrocarbon-impacted environments (reviewed in Gieg et al., 2014). The cultured Syntrophus sp. that are related to the organism identified here are characterized by their ability to degrade benzoate in syntrophic cooperation with methanogens (Wallraubenstein et al., 1995; Jackson et al., 1999). Members of the Syntrophus/Smithella genera have previously been implicated in alkane and benzene activation under methanogenic conditions, thus these
organisms have a demonstrated potential for methanogenic hydrocarbon metabolism (Zengler et al., 1999; Jones et al., 2008; Sakai et al., 2009; Gray et al., 2011; Cheng et al., 2013). Our results indicate that Syntrophaceae incorporated carbon from toluene and responded to toluene exposure at a later time than Desulfosporosinus sp. This suggests either that Syntrophaceae has a slower response to toluene exposure than Desulfosporosinus sp. or that it degrades a compound produced during toluene metabolism. One possibility is that the Syntrophaceae phylotype identified here competes with Desulfosporosinus sp. for toluene, but responds more slowly. Alternatively it may be involved in the degradation of benzoate, or could play a role further downstream, potentially degrading smaller fatty acids into methanogenic substrates, or carrying out acetate oxidation, roles which have previously been ascribed to other members of the Syntrophaceae such as Smithella propionica and Desulfovibacca acetoxidans (Liu et al., 1999; Oude Elferink et al., 1999). The strong response of Syntrophaceae 16S rRNA transcription in response to toluene exposure suggests that it plays an important role in toluene metabolism in this culture (Fig. 3).

Members of Desulfovibrio sp. are commonly found in a variety of strictly anaerobic oil-impacted environments such as oil reservoirs and enrichment cultures derived from contaminated sites and oil production waters (Grabowski et al., 2005; Fowler et al., 2012; Mbadinga et al., 2012; Callbeck et al., 2013). Though they seem to be ubiquitous in these environments, their specific role has not been elucidated although they have never been associated with hydrocarbon degradation directly. As the Desulfovibrio sp. identified here did not incorporate labelled carbon until after Desulfosporosinus sp., and had a smaller and slower response to toluene exposure, it is proposed that it is likely involved in reactions in downstream toluene metabolism (Figs 1 and 3). It can be speculated that Desulfovibrio sp. plays a role in fatty acid metabolism and/or the consumption of hydrogen as these are typical of the metabolisms of Desulfovibrio spp. under sulphate-reducing and syntrophic conditions (Walker et al., 2009; Plugge et al., 2010). Also interesting to note is the microdiversity that exists within the Desulfovibrio lineage in this culture. Multiple bands affiliated with Desulfovibrio sp. in DGGE analysis (Table S1), suggesting that these are closely related, yet slightly different members of this clade.

A member of the phylum Chloroflexi became labelled in this culture at a later time point (day 7), suggesting that it may use a product generated by one of the organisms described above and thus incorporate label from toluene (Fig. 1). Organisms that were present in DGGE analysis but did not become substantially labelled in 7 days included two organisms related to Geobacter sp. and the Desulfbacterales, a member of the Spirochaetes and a Clostridium sp. whose activity did not increase significantly when exposed to toluene, and did not appear to incorporate carbon from toluene within 7 days. The roles of these unlabelled organisms are elusive. It is possible that some organisms may play supportive roles as has been shown in other cultures, such as maintaining a low redox potential or providing co-factors and vitamins (Hug et al., 2012). However, organisms carrying out such roles would need to conserve sufficient energy to persist in the culture. Longer incubation times may have revealed the incorporation of 13C-label into these organisms.
An important observation made with regards to the results of RNA-SIP experiment was that shifts in buoyant density were not dramatic, and that some of the labelled organisms formed strong bands in the light fraction as well as the heavy fractions of the labelled samples. The primary cause of this is likely the concomitant incorporation of both 13C from toluene as well as unlabelled carbon, most likely derived from bicarbonate, over the time course. Carbon fixation during anaerobic hydrocarbon metabolism was previously proposed (Winderl et al., 2010), and has recently been demonstrated by SIP with labelled bicarbonate (Taubert et al., 2012). Such carbon fixation results in a dilution of the 13C label and smaller increases in buoyant density in those organisms incorporating both labelled and unlabelled carbon, thereby hampering the identification of organisms degrading the labelled substrate during SIP analysis.

Meghanolic communities are characterized by the complex energetic relationships that exist among their members. This work highlights these complex syntrophic interactions. On the basis of our data with this toluene-degrading enrichment culture, we can propose a working model for how toluene is metabolized by the consortium (Fig. 4). The direct attack on toluene appears to be catalysed by Desulfosporosinus sp. We hypothesize that Desulfosporosinus sp. degrades toluene into smaller molecules such as cyclohexane carboxyl-CoA, pimelyl-CoA and glutaryl-CoA, which were previously detected in culture fluids (Fowler et al., 2012) (Fig. 4). Our results do not preclude the possibility that a member of the Syntrophaceae may also be capable of toluene activation in this culture. The subsequent steps involved in methanogenic toluene metabolism in this culture are unclear. Downstream metabolism of benzyol-CoA beyond glutaryl-CoA is thought to proceed via β-oxidation in pure cultures (Harrison & Harwood, 2005); however, it is also possible that syntrophic metabolism proceeds via a different route, such as via fermentation to volatile fatty acids or alcohols which might be catalysed by a member of the Syntrophaceae or Syntrophobacterales or Syntrophobacteraceae (Fig. 4). The Chloroflexi that also became labelled could be carrying out syntrophic acetate oxidation, or could be involved in the fermentation of another labelled intermediate. The remaining organisms that were present in the culture but did not become strongly labelled within 7 days such as Clostridium sp., could be acting acetogenically, or scavenging H2 and/or other products produced by the culture, carrying out syntrophic acetate oxidation (which would result in only low level labelling if acetate is derived from 13C2-toluene) or they may be involved in subsequent reactions in the conversion of the intermediates shown (Fig. 4). Due to previous observations revealing the presence of hydrogenotrophic and acetoclastic methanogens in this culture, as well as the close to stoichiometric conversion of toluene to methane (Fowler et al., 2012), it is likely that the bacteria present in this culture convert the intermediates shown into methanogenic substrates such as acetate, CO2 and H2.

In summary, this study has confirmed the importance of Desulfosporosinus sp. in anaerobic toluene degradation under methanogenic conditions and has also revealed a role for Syntrophaceae and Desulfovibrionales in methanogenic toluene degradation. Our results also indicate that in slow growing cultures such as these, examining the RNA content of a culture is an effective and sensitive way to unravel community and metabolic interactions. Lastly, the complex community structure revealed in this study on a stable, long standing laboratory enrichment culture highlights the extraordinary complexity of hydrocarbon-metabolizing methanogenic communities.

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References


**Supporting Information**

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

**Table S1.** Bacterial diversity present in a methanogenic toluene-degrading culture determined by DGGE and similarity to cultured and uncultured microbes.