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Research Article

RESEARCH ARTICLE

# DNA stable-isotope probing of oil sands tailings pond enrichment cultures reveals different key players for toluene degradation under methanogenic and sulfidogenic conditions

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**One sentence summary:** An uncultivated *Desulfosporosinus* sp. degraded toluene in oil sands tailings enrichment cultures incubated under methanogenic conditions but, surprisingly, was outcompeted by a *Desulfobulbaceae* member under sulfate-reducing conditions.

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## ABSTRACT

Oil sands tailings ponds are anaerobic repositories of fluid wastes produced by extraction of bitumen from oil sands ores. Diverse indigenous microbiota biodegrade hydrocarbons (including toluene) *in situ*, producing methane, carbon dioxide and/or hydrogen sulfide, depending on electron acceptor availability. Stable-isotope probing of cultures enriched from tailings associated specific taxa and functional genes to $^{13}C_6$- and $^{12}C_7$-toluene degradation under methanogenic and sulfate-reducing conditions. Total DNA was subjected to isopycnic ultracentrifugation followed by gradient fraction analysis using terminal restriction fragment length polymorphism (T-RFLP) and construction of 16S rRNA, benzylsuccinate synthase (*bssA*) and dissimilatory sulfite reductase (*dsrB*) gene clone libraries. T-RFLP analysis plus sequencing and *in silico* digestion of cloned taxonomic and functional genes revealed that Clostridiales, particularly *Desulfosporosinus* (136 bp T-RF) contained *bssA* genes and were key toluene degraders during methanogenesis dominated by *Methanosaeta*. Deltaproteobacterial *Desulfobulbaceae* (157 bp T-RF) became dominant under sulfidogenic conditions, likely because the *Desulfosporosinus* T-RF 136 apparently lacks *dsrB* and therefore, unlike its close relatives, is presumed incapable of dissimilatory sulfate reduction. We infer incomplete oxidation of toluene by *Desulfosporosinus* in syntrophic association with *Methanosaeta* under methanogenic conditions, and complete toluene oxidation by *Desulfobulbaceae* during sulfate reduction.

**Keywords:** anaerobic toluene degradation; tailings; methanogenesis; sulfate reduction; syntrophy; fumarate addition; benzylsuccinate synthase

## INTRODUCTION

Oil sands tailings ponds (OSTP; historically, 'tar sands' tailings ponds) in Northeastern Alberta, Canada are repositories for the fluid wastes generated by extraction of bitumen from surface-mined oil sands ores. Collectively, the ponds currently cover a surface area of >180 $km^2$ and contain nearly 1 billion $m^3$ of tailings (Alberta Energy Regulator 2013) comprising colloidal suspensions of water, sand, clays, unrecovered bitumen and light hydrocarbon solvents used in bitumen extraction. OSTP

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typically are stratified, with an oxic to suboxic water layer at the surface (Stasik and Wendt-Potthoff 2014) overlying the anaerobic semi-fluid suspension of solids and hydrocarbons, typically >30 m deep. Because of their size and environmental footprint, OSTP are of increasing scientific and public interest (Gosselin *et al.* 2010).

All OSTP examined to date harbor diverse, active microbial communities (Penner and Foght 2010; Ramos-Padrón *et al.* 2011; An *et al.* 2013) that influence the properties of the tailings and biogeochemistry of the ponds through redox reactions (Holowenko, MacKinnon and Fedorak 2000; Fedorak *et al.* 2003; Stasik *et al.* 2014). Microbial activity in most OSTP appears to be supported primarily by anaerobic biodegradation of certain solvent components (Siddique *et al.* 2007; Abu Laban *et al.* 2015c). Methanogenic activity, in the form of biogenic greenhouse gas emissions ($CH_4$ and $CO_2$), is most commonly observed but sulfidogenic activity is prevalent in some OSTP where gypsum (calcium sulfate) has been added as a tailings processing aid (Ramos-Padrón *et al.* 2011).

In Mildred Lake Settling Basin, the largest OSTP operated by Syncrude Canada Ltd., the extraction solvent is naphtha, a complex mixture of light hydrocarbons ($\sim C_6$–$C_{10}$) comprising *n-*, *iso-* and *cyclo*-alkanes and monoaromatics. Because toluene is a major organic constituent of naphtha in the fresh tailings entering this OSTP, and is among the most rapidly degraded hydrocarbons in those tailings (Siddique *et al.* 2007), it supports methanogenesis *in situ*. Also, since this OSTP historically received gypsum and because other companies' OSTP that receive both naphtha and gypsum are sulfidogenic (Ramos-Padrón *et al.* 2011), we sought to determine the impact of different electron-accepting processes on hydrocarbon metabolism by tailings microbiota, specifically the biodegradation of toluene as a model for monoaromatics in OSTPs.

Recent studies of anaerobic toluene biodegradation under sulfate-reducing and methanogenic conditions have detected Clostridiales including *Peptococcaceae* (particularly *Desulfosporosinus*) and/or *Desulfobulbaceae* (Pilloni *et al.* 2011; Fowler *et al.* 2012, 2014; Sun and Cupples 2012; Sun, Sun and Cupples 2014). We hypothesized that these taxa, which are indigenous to several OSTP (An *et al.* 2013), might also dominate methanogenic OSTP enrichment cultures incubated with toluene. However, discerning which taxa would prevail in response to sulfate as an electron acceptor was of interest since Liu *et al.* (2004) reported that toluene-degrading *Desulfosporosinus* sp. Y5 could utilize diverse inorganic electron acceptors including sulfate.

Therefore, we used DNA stable-isotope probing (SIP) to target the active taxa involved in toluene degradation by incubating cultures enriched from Mildred Lake Settling Basin tailings with $^{13}C_6$- or $^{12}C_7$-toluene under sulfate-reducing and methanogenic conditions. Metagenomic DNA extracted from labeled and unlabeled fractions of buoyant density gradients was analyzed by using terminal restriction fragment length polymorphism (T-RFLP) as well as by constructing and sequencing clone libraries of taxonomic (16S rRNA) and functional (*assA* and *dsrB*) genes. Such information would enhance understanding of microbial communities accomplishing aromatic hydrocarbon degradation in anaerobic environments.

## METHODS AND MATERIALS

### Enrichment culture growth conditions

Methanogenic and sulfate-reducing enrichment cultures were established using anaerobic mature fine tailings from the Mildred Lake Settling Basin (57°04′12.0″N, 111°38′24.0″W) OSTP in Northeastern Alberta, Canada. These tailings (≥30 wt% solids) typically have naphtha contents of 0.03–0.25 wt% (Siddique *et al.* 2007), circumneutral pH, temperatures of 12–22°C *in situ* and pore water concentrations of 0.1–36 mg $L^{-1}$ sulfate and <0.04 mg $L^{-1}$ nitrate + nitrite (Penner and Foght 2010). The initial culture, established in a 158-ml sealed serum bottle, was enriched by mixing 50 ml tailings with 50 ml anaerobic mineral fresh water medium (Widdel and Bak 1992) plus a mixture of 0.5 mM each of benzene, toluene, ethylbenzene and xylenes under a headspace of $N_2$ and $CO_2$ (80:20 v/v) and incubated at 28°C in the dark, monitoring the headspace for methane production. This primary enrichment culture was subsequently subcultured (10 vol% transfer) twice this way, but with toluene as sole added carbon source. Six replicate bottles of the second transfer were pooled and used as 10 vol% inoculum for the main experiment comprising 48 replicate cultures in the same medium. The bottles were divided into four sets of 12: two sets received 5 $\mu$L (~0.5 mM final concentration) $^{13}C_6$-toluene (99% purity, Cambridge Isotopes, Tewksbury, MA) and two sets received 5 $\mu$L (~0.5 mM) $^{12}C_7$-toluene (99% purity, Sigma-Aldrich, Oakville, ON, Canada). One set with each isotope additionally received 10 mM $Na_2SO_4$ (sulfate-reducing conditions); the other received no additional electron acceptor (methanogenic conditions). Two parallel live control cultures were prepared that received neither toluene nor sulfate. All cultures were reduced with 1 mM $Na_2S$ and incubated stationary at 28°C in the dark.

### Analytical procedures

Methane production was monitored at intervals during incubation by removing 0.1 ml of headspace gas from bottles, using sterile needle and 0.5 ml syringe, and directly injecting into a Hewlett Packard 5700A gas chromatograph (GC) equipped with a flame ionization detector as previously described (Holowenko, MacKinnon and Fedorak 2000). Soluble sulfide concentrations in sulfate-admended cultures were assessed spectrophotometrically using a colorimetric methylene blue assay (Cline 1969).

To monitor residual toluene concentrations in the sealed bottles, 100 $\mu$L of headspace was directly injected into a gas chromatograph-mass spectrometer (GC-MS; Agilent 6890N with a model 5973 inert mass selective detector) fitted with a HP-5MS capillary column (30 m length, 0.25 mm inner diameter, 0.25 $\mu$m film thickness; Agilent, USA). The injector temperature was 250°C, the carrier gas was helium at a flow rate of 1 ml $min^{-1}$ and split/splitless ratio 1:10. The oven temperature program was 40°C for 5 min followed by an increase of 5°C $min^{-1}$ to 85°C. The standard curve for toluene concentration was calculated by headspace analysis of parallel abiotic samples prepared in serum bottles with mineral medium containing 0.1, 0.2, 0.3 or 0.5 mM toluene plus heat-sterilized tailings. The GC-MS was operated in single ion monitoring mode with *m/z* 92 for $^{12}C_7$-toluene and *m/z* 98 for $^{13}C_6$-toluene.

Total $^{13}CO_2$ + $^{13}CH_4$ production was determined by measuring the $\delta^{13}C$ stable isotope ratio of gases in the headspace. A total of 500 $\mu$L of headspace removed from the sample bottle were diluted in a sealed 5-ml serum vial with helium headspace. A total of 100 $\mu$L of the diluted gas sample were injected onto a Conflo III & Continuous Flow DeltaPlus Advantage Isotope Ratio Mass Spectrometer (CF-IRMS; ThermoFinnigan, Bremen, Germany), where intensities of *m/z* 46, 45 and 44 for $CO_2$ were quantified by using a thermal conductivity detector. Internal standards, calibrated against the international reference scale

for carbon (V-PeeDee Belemnite; VPDB) were used to calculate sample isotopic results.

## DNA extraction and density fractionation

During incubation, four sampling times were chosen to monitor $^{13}$C-DNA appearance, based on toluene depletion and production of end products (methane or sulfide). At each time point, biomass from three 75-ml culture bottles was harvested individually for total genomic DNA extraction by centrifugation (3700 $\times$ *g* for 30 min at 4°C). DNA was extracted using modified protocols based on Lueders, Manefield and Friedrich (2004) and Gabor, de Vries and Janssen (2003). The pellet was suspended in 750 $\mu$L PTN buffer (120 mM sodium phosphate, 125 mM Tris-HCl, 0.25 mM NaCl; pH 8) and transferred to sterile 2-ml screw-cap microfuge tubes (Fisherbrand, Fisher Scientific) containing 0.5 g each of 0.1 mm and 2.3 mm zirconia/silica beads (BioSpec Products, Bartlesville OK). Then, 40 $\mu$L of lysozyme (50 mg ml$^{-1}$) and 10 $\mu$L of Proteinase K (10 mg ml$^{-1}$) were added and incubated at 37°C for 15 min, during which the samples were hand-mixed every 2 min. A total of 100 $\mu$L of 20% SDS was added and incubated at 65°C for 15 min with mixing at 2 min intervals. After addition of 100 $\mu$L phenol: chloroform: isoamyl alcohol (25:24:1) the samples were agitated for 45 s at 6.0 m s$^{-1}$ in a FastPrep Cell Disrupter (Bio 101 Systems, Thermo, Milford, USA). The bead-beating tubes were microcentrifuged at 7500 rpm for 5 min at 4°C and the supernatants were transferred to new tubes. Thereafter, another 300 $\mu$L PTN buffer were added to each tube followed by another cycle of bead beating for 20 s at 6.5 m s$^{-1}$ and microcentrifugation. Both supernatants were pooled to achieve 900 $\mu$L total, then 1 vol of phenol:chloroform:isoamyl alcohol was added and shaken vigorously before microcentrifugation at 14 000 rpm and 4°C for 4 min. The upper layer was transferred to a new tube containing 1 vol chloroform:isoamyl alcohol (24:1), the contents mixed, then microcentrifuged again. The supernatant was added to 2 vol 30% polyethylene glycol buffer and incubated overnight at 4°C. Precipitated DNA was recovered by microcentrifugation for 30 min at room temperature, decanting the polyethylene glycol, adding 150 $\mu$L 70% ethanol (at −20°C) and microcentrifuging again at 4°C. After decanting the ethanol, the samples were air dried for 30 min at room temperature and DNA was dissolved in 40 $\mu$L DNA-free Milli-Q water (Millipore, Billerica MA). To reduce variability, triplicate aliquots were individually extracted twice sequentially and finally the six DNA extracts from each sample were pooled. Parallel reagent-only extractions were free from DNA contamination, as shown by PCR (data not shown).

The DNA-SIP cesium chloride fractionation protocol described by Lueders (2010) was performed on DNA recovered at each of two time points corresponding to 50–60% and 80–100% toluene degradation. Briefly, total genomic DNA (1–4 $\mu$g) was added to 15-ml Falcon tubes (BD Biosciences, Missisauga, ON) and mixed with gradient buffer to 900 $\mu$L total volume, followed by addition of 5 ml of cesium chloride solution (~1.84 g ml$^{-1}$; Ambion, molecular biology grade). The refractive index was measured using 75 $\mu$L aliquots to determine and adjust density prior to centrifugation, where the temperature-corrected refractive index readings should be 1.4040 ± 0.0002 (~1.71 g ml$^{-1}$ cesium chloride). The mixture was transferred to 5.1 ml polyallomer QuickSeal tubes (Beckman, Palo Alto, CA) using sterile syringe and 1.2 mm needle to avoid introducing air bubbles. The tubes were heat sealed and centrifuged using a VTi 65.2 rotor in a Beckman Optima™ LE-80K Ultracentrifuge for 65 h at 165 000 $\times$ *g*, 20°C, with brakes off below 5000 rpm during deceleration. After centrifugation, each gradient was fractionated from the bottom of the tube using a peristaltic pump operating at 1 ml min$^{-1}$ on continuous mode to collect 12–14 fractions (~380–400 $\mu$L each) at 25 s intervals. The refractory index of each fraction was measured to calculate buoyant density, followed by nucleic acid precipitation, as described by Lueders (2010).

## Analysis of DNA in buoyant density gradient fractions

Total bacterial 16S rRNA gene copies in gradient fractions were quantified using primers Ba519f/Ba907r (Lueders, Manefield and Friedrich 2004) and Sybr Green qPCR (Applied Biosystem 7500 Fast Real-Time PCR, Burlington, ON), in parallel with a high sensitivity fluorometric quantification assay (Invitrogen Qubit® 1.0 Fluorometer, Burlington, ON).

T-RFLP analysis was performed on subsamples of $^{12}$C and $^{13}$C gradient fractions. Bacterial and archaeal 16S rRNA genes were amplified separately using primers Ba27f-FAM/Ba907r and Ar109f/Ar912rt-FAM (Lueders, Pommerenke and Friedrich 2004) and PCR amplicons were digested using MspI and Taq1 for Bacteria and Archaea, respectively (Lueders, Pommerenke and Friedrich 2004). Digested amplicons were desalted using DyeEx® 2.0 Spin Kit (Qiagen, Toronto, Canada) and analyzed as previously described (Winderl, Schaefer and Lueders 2007) using GeneScan™ 1200 LIZ® dye size standard (Applied Biosystems®, Burlington, Ontario) to calculate T-RF sizes.

Partial 16S rRNA gene sequences for clone library construction were amplified using the bacterial and archaeal primers Ba27F/1492R (Weisburg *et al.* 1991) and Ar21f/Ar1383r (DeLong 1992; Weber *et al.* 2001), respectively; PCR reactions, cloning and sequencing were performed per Winderl, Schaefer and Lueders (2007). For clone sequencing, 40–48 clones were picked randomly from the bacterial 16S rRNA gene libraries and 20–25 from archaeal libraries. The sequences were compared to those in GenBank (NCBI; www.ncbi.nlm.nih.gov/, 13 April 2015, date last accessed) using BlastN for presumptive phylogenetic identification. Sequences of ~800 bp were aligned with their nearest neighbors using ClustalW, and neighbor-joining and maximum likelihood phylogenic trees were computed using default parameters implemented in MEGA5 software (Tamura *et al.* 2011).

Partial *bssA* gene sequences (~794 bp) were amplified from DNA in gradient fractions using primers 7772f/8546r and previously described PCR conditions (Winderl, Schaefer and Lueders 2007); partial *dsrB* gene sequences of ~370–390 bp were amplified using primers dsrp2060F/dsr4R and PCR conditions described by Dar *et al.* (2007). Cloning and sequencing of both gene amplicons were performed according to Winderl, Schaefer and Lueders (2007). Translated sequences of benzylsuccinate synthase subunit A (BssA) and dissimilatory sulfite reductase subunit B (DsrB) of ~258 and ~125 amino acids, respectively, were compared with closely related sequences using BLASTP (blast.ncbi.nlm.nih.gov, 8 April 2015, date last accessed), followed by alignment using ClustalW (www.genome.jp/tools/clustalw/, 8 April 2015, date last accessed) and phylogenic trees were computed as described above.

Amplified DNA sequences presented in this manuscript have been deposited in GenBank under the following accession numbers: bacterial 16S rRNA genes (KJ406578–KJ406683), archaeal 16S rRNA genes (KJ406660–KJ406683), *bssA*-like genes (KJ397988–KJ398022) and *dsrB* genes (KJ398023–KJ398065). Draft genomes of '*Desulfosporosinus* sp. Tol-M', assembled from the metagenome of the methanogenic $^{13}C_6$-toluene culture and of '*Desulfobulbaceae* bacterium Tol-SR' from the sulfidogenic

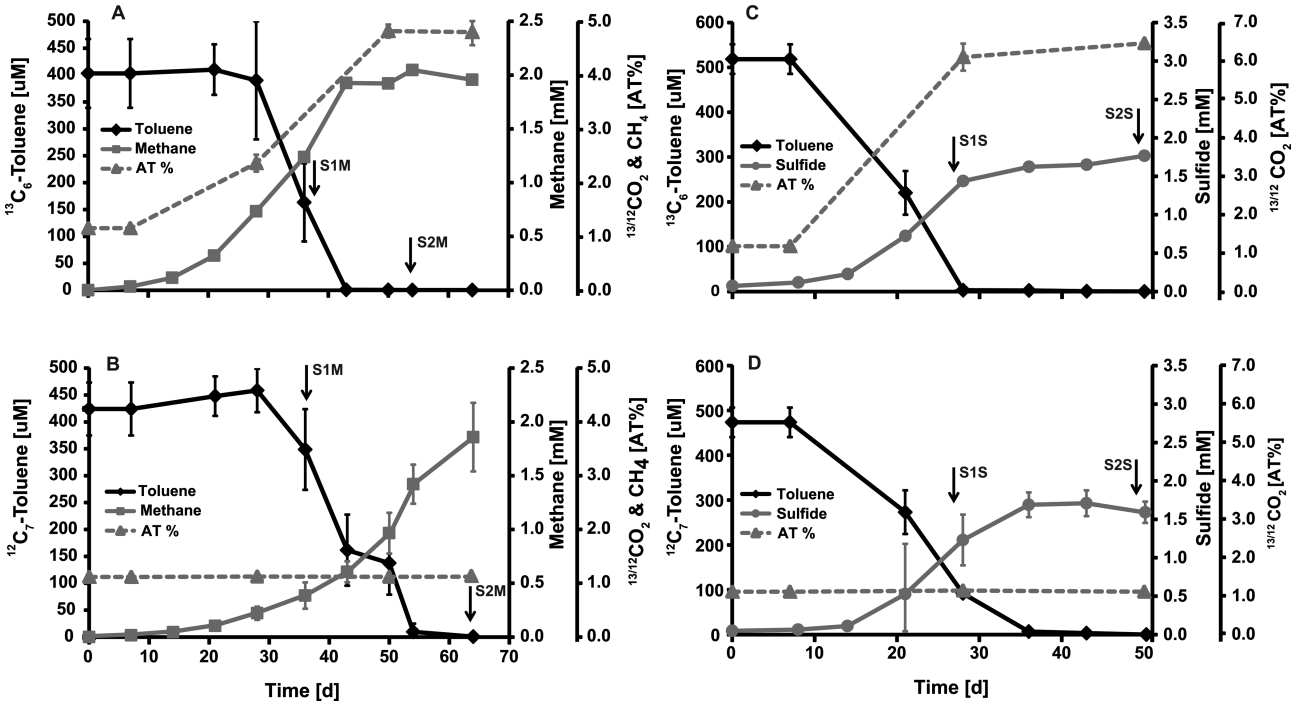


**Figure 1.** Anaerobic biodegradation of toluene by replicate oil sands tailings enrichment cultures incubated under methanogenic or sulfidogenic conditions. (**A**) and (**B**): methanogenic degradation of (A) $^{13}C_6$-toluene or (B) $^{12}C_7$-toluene (black diamonds); cumulative production of methane (grey squares); $^{13}C/^{12}C$ isotopic enrichment of $^{13}CH_4 + {}^{13}CO_2$ in headspace, expressed as AT% (grey triangles and dashed lines). (**C** and **D**): sulfidogenic degradation of (C) $^{13}C_6$-toluene or (D) $^{12}C_7$-toluene (black diamonds); sulfide production (grey circles); $^{13}C/^{12}C$ isotopic enrichment of $^{13}CO_2$ (grey triangles and dashed lines). The $^{13}C/^{12}C$ ratios in panels B and D represent natural isotope abundance, which is unchanged in cultures incubated with unlabeled toluene. Data points are the mean of triplicate cultures and error bars, where visible, represent one standard deviation. S1M, S2M, S1S and S2S (arrows) indicate the sample times chosen for harvesting biomass from triplicate cultures.

culture, have been deposited with DDBJ/EMBL/GenBank under accession numbers JQID00000000 (Abu Laban *et al.* 2015a) and JROS00000000 (Abu Laban *et al.* 2015b), respectively. Metagenome sequences of *bssA*-like genes (KP009582 and KP009583) and *dsrB* (KP009586) also have been deposited with NCBI.

## RESULTS

### Degradation of $^{13}C_6$- and $^{12}C_7$-toluene under methanogenic and sulfidogenic conditions

Enrichment cultures established from oil sands tailings completely degraded $^{13}C_6$- and $^{12}C_7$-toluene under both methanogenic and sulfate-reducing conditions within 30–55 d (Fig. 1). Accumulation of methane and particularly isotopically-enriched $^{13}CH_4 + {}^{13}CO_2$ was observed during incubation with $^{13}C_6$-toluene (Fig. 1A) in the absence of sulfate. An equivalent mass of methane was produced from the $^{12}C_7$-toluene cultures, with no change in the $^{13/12}(CO_2 + CH_4)$ natural isotopic abundance ratio (Fig. 1B). Negligible methane was produced by the live control cultures that did not receive toluene (data not shown), as endogenous substrates had been diluted during passage. The sulfidogenic cultures exhibited a shorter lag time before onset of toluene degradation, after which $^{13}CO_2$ production paralleled sulfide accumulation (Fig. 1C).

The electron balance for methanogenesis (Equation 1; Heider *et al.* 1999) predicts recovery of ~80% of $^{13}C_6$-toluene as $^{13}CO_2 + {}^{13}CH_4$; this calculation agrees with measurement of ~5 AT% (atomic per cent) $^{13}C$ in the headspace gas near the end of degradation, i.e., ~83% of the maximum ~6 AT% expected. Likewise, sulfate reduction yielded 86% of the maximum $^{13}CO_2$ expected from $^{13}C_6$-toluene, and measurement of ~72% of the electrons in toluene as sulfide (Eq. 2; Heider *et al.* 1999).

$$C_7H_8 + 7.5H_2O \rightarrow 2.5HCO_3^- + 4.5CH_4 + 2.5H^+$$
$$\Delta G^{o\prime} = -131\,\text{kJ mol}^{-1}\text{ toluene} \quad (1)$$

$$C_7H_8 + 4.5SO_4^{2-} + 2H^+ + 3H_2O \rightarrow 7HCO_3^- + 4.5H_2S$$
$$\Delta G^{o\prime} = -205\,\text{kJ mol}^{-1}\text{ toluene} \quad (2)$$

Thus, the predicted and measured stoichiometry agree under both electron-accepting conditions and support our observation of complete oxidation of toluene to $CO_2 \pm CH_4$, with the remaining ~15–20% of $^{13}C$-carbon presumably being incorporated into biomass (e.g. DNA) and/or present as water-soluble intermediates.

### Different T-RFs dominate under methanogenic and sulfidogenic conditions

During incubation, we sampled the four sets of replicate cultures at 50–60% or 100% toluene depletion in methanogenic cultures (time points S1M and S2M, respectively) and 80% or 100% toluene depletion in sulfidogenic cultures (S1S and S2S; indicated by arrows in Fig. 1). These samples were subjected to total DNA extraction, isopycnic ultracentrifugation, fractionation and qPCR to determine the relative abundance of 16S rRNA genes in each fraction, normalized to the maximum copy number. The second sampling points (S2M and S2S) were selected for more detailed

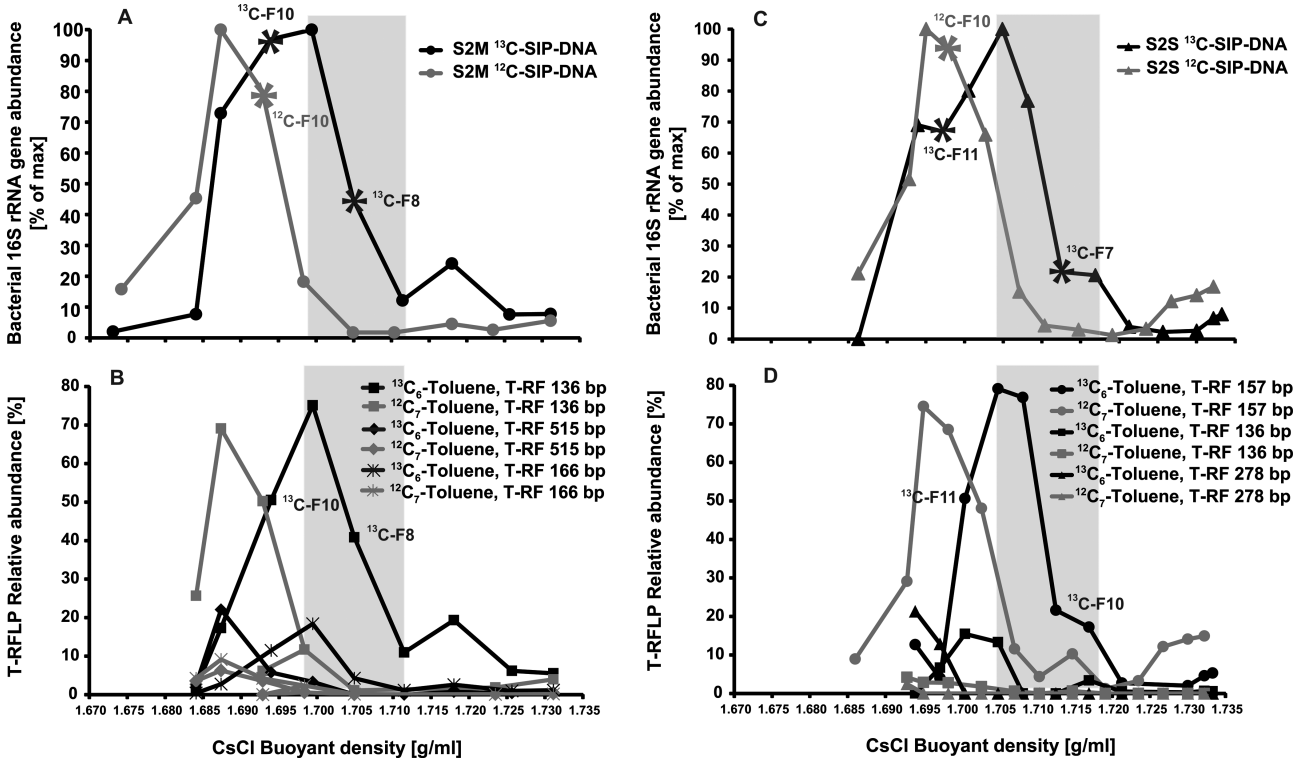


**Figure 2.** Upper panels: Quantitative distribution of total 16S rRNA genes in SIP fractions containing $^{13}$C- and $^{12}$C-DNA extracted at time points S2M and S2S, determined using qPCR and expressed as percentage of maximum abundance. Lower panels: Distribution of the three most abundant T-RFs in methanogenic (**A** and **B**) or sulfidogenic (**C** and **D**) culture fractions. Fractions chosen for detailed analysis are indicated with asterisks. The shaded areas indicate fractions considered to be enriched in $^{13}$C-DNA from active (primary) toluene degraders.

study because they clearly showed $^{13}$C incorporation into DNA by correlation with increased density and 16S rRNA gene abundance (Fig. 2A and C).

Selected fractions were subjected to T-RFLP analysis and preparation of clone libraries of taxonomic and functional genes to reveal the key players in toluene degradation. From the S2M methanogenic gradients, we selected heavy fraction $^{13}$C-F8 (Fig. 2A) to represent taxa actively incorporating $^{13}$C via primary degradation of toluene (and/or $^{13}$C-metabolites via secondary labeling). The corresponding $^{12}$C-F10 fraction (Fig. 2B) from the $^{12}C_7$-toluene gradient (in which there is no heavy DNA) was selected to represent the most abundant – and hence the most active – members of the unlabeled methanogenic culture. In comparison, the light $^{13}$C-F10 fraction presumably represents accessory taxa that had not significantly incorporated the heavy isotope by time point S2M. In the two sulfate-reducing gradients, fractions $^{13}$C-F7 and $^{12}$C-F10 were chosen to highlight taxa actively incorporating toluene whereas the light $^{13}$C-F11 fraction was selected to represent ancillary taxa (Fig. 2C and D).

To examine the diversity of bacterial phylotypes, T-RFs of cloned 16S rRNA genes from $^{13}$C-DNA fractions were compared to those of corresponding $^{12}$C-DNA fractions (Fig. S1, Supporting Information). To further resolve incorporation of the $^{13}$C-DNA label, relative gene abundance was calculated for the three most dominant T-RFs in each fraction and plotted versus buoyant density (Fig. 2B and D). T-RF 136 bp dominated both 'active' fractions from the methanogenic cultures, representing 40–75% of the maximum bacterial 16S rRNA gene abundance in fractions $^{13}$C-F8 to $^{13}$C-F10 and 25–70% in fractions $^{12}$C-F10 to $^{12}$C-F12 (Fig. 2B). Additional T-RFs were also detected in all gradient fractions (e.g. 166 and 515 bp; Fig. 2B and Fig. S1B, Supporting Information) but in much lower proportions. In contrast, in the sulfidogenic culture, T-RF 157 replaced T-RF 136 as the dominant T-RF in active fractions ($^{13}$C-F8 to F10 and $^{12}$C-F10 to F12; Fig. 2D). Here also additional T-RFs, including those of 136, 278 and 515 bp, were present in S2S fractions (Fig. 2D and Fig. S1D, Supporting Information) as minor members. The common but unlabeled taxa present at low abundance under both culture conditions may represent a 'core microbiota' not directly involved in the primary steps of toluene degradation.

Archaeal T-RFLP profiles of the methanogenic culture active fractions were dominated by T-RF 280 (Fig. S2, Supporting Information), suggesting that it is the primary consumer of toluene metabolites under methanogenic conditions. Of the other T-RFs detected (e.g. 181 and 388 bp), only T-RF 181 was markedly enriched in $^{13}$C fractions.

## Key toluene degraders in methanogenic cultures

The identity of prominent bacterial T-RFs in methanogenic $^{13}$C-DNA gradients (Fig. 2) was established using sequenced 16S rRNA clones, BLAST queries of GenBank and the curated RDP database (rdp.cme.msu.edu, release 11.3) (Table S1, Supporting Information) plus affiliation within a phylogenetic tree (Fig. S3, Supporting Information). *In silico* digestion of cloned sequences additionally generated synthetic T-RFs (Tables S1 and S2, Supporting Information) with which to identify the measured T-RFs discussed above. Notably, the length of *in silico* T-RF digests is typically 4–7 bp larger than that of measured T-RFs due to the different fluorophore used on the commercial molecular weight ladder that affects fragment mobility and causes the measured T-RF size to be underestimated; this systemic discrepancy

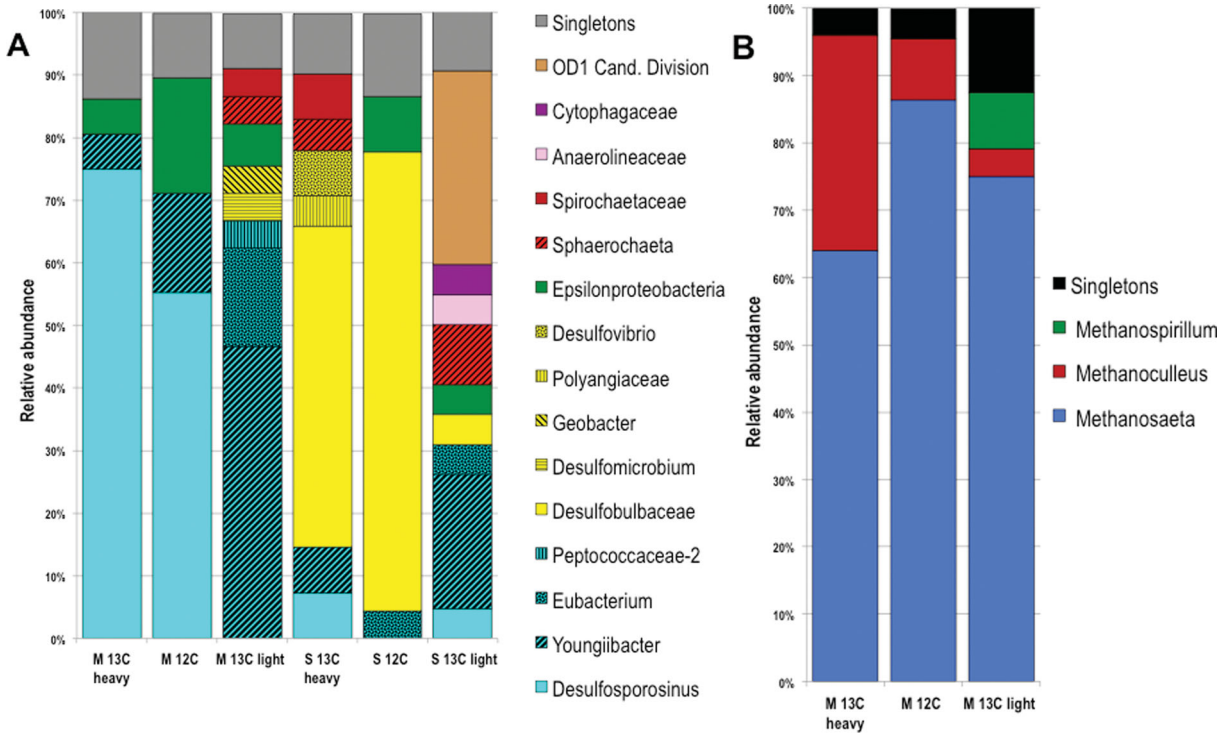


**Figure 3.** Composition of (**A**) bacterial and (**B**) archaeal 16S rRNA gene clone libraries constructed using selected SIP fractions (indicated by asterisks in Fig. 2) from methanogenic (M) and sulfidogenic (S) cultures at time points S2M and S2S. Taxon abundance is expressed as percentage of total clones in each library, with the lowest confident taxonomic level shown. Bacterial clones affiliated with different families or genera within the Clostridiales (Firmicutes) are shown in blue; Deltaproteobacteria in yellow and Spirochaetales in red; single clones, grouped as 'singletons' are shown in grey.

cannot be removed using a simple mathematical correction factor because the magnitude of the effect is not constant and depends on the T-RF lengths (Schütte *et al.* 2008). Therefore, both observed and virtual T-RF sizes are reported in Tables S1 and S2 (Supporting Information).

Members of the Firmicutes dominated the three clone libraries constructed at sample time S2M. In particular, clones affiliated with family *Peptococcaceae* (order Clostridiales) having closest matches to *Desulfosporosinus* spp. represented 67 and 50% of the clones from 'active' fractions $^{13}$C-F8 and $^{12}$C-F10, respectively (Fig. 3A and Fig. S3, Supporting Information) and their *in silico* T-RFs (Table S1, Supporting Information) corresponded to the dominant T-RF 136 shown in Fig. 2B. Therefore, *Desulfosporosinus* T-RF 136 is interpreted to represent the key taxon initiating toluene degradation in these methanogenic cultures. Clostridiales clones affiliated with *Youngiibacter* (T-RF 515; Table S1, Supporting Information) increased in proportion from the heavy $^{13}$C-F10 fraction to the unlabeled $^{12}$C-F10 fraction and dominated the light $^{13}$C-F10 fraction (Fig. 3A), suggesting that *Youngiibacter* T-RF 515 is an ancillary fermenter during methanogenic toluene degradation along with two other Clostridiales-related clones (*Peptococcaceae*-2 T-RF 172 and *Eubacterium* T-RF 161, detected only in the light $^{13}$C-F10 fraction; Fig. 3A). An Epsilonproteobacterium (T-RF 462) was detected at low abundance (5–18%) in all clone libraries, including as a single clone from the heavy $^{13}$C-F7 fraction, suggesting that it also belongs to the 'core' microbiota and plays a supporting role in the community regardless of electron acceptor.

The majority (~65–85%) of archaeal clones in all three S2M fractions (Fig. 3B) were 100% similar to *Methanosaeta* spp. (T-RF 280; Table S2 and Fig. S4, Supporting Information), accompanied by lesser proportions (~5–30%) of *Methanoculleus* (T-RF 181). Small proportions of *Methanospirillum* (T-RF 388) and single clones of other genera were detected in the light $^{13}$C-F10 fraction. Although the archaeal clone libraries were small (22–24 clones each) and may not have revealed the full diversity of Archaea in the cultures, it is clear that acetoclastic methanogens are predominant, with minor proportions of hydrogenotrophic or other methanogens.

## Key toluene degraders under sulfidogenic conditions

In the sulfate-reducing cultures, ~50–70% of clones were affiliated with the Deltaproteobacterial family *Desulfobulbaceae* (e.g. clone *Desulfocapsa* JQ086868) in both the 'active' $^{13}$C-F7 and $^{12}$C-F10 fractions, but were minor components (<5%) of the light $^{13}$C-F11 fraction clone library (Fig. 3A and Fig. S3, Supporting Information) in sample S2S. *In silico* digestion of the *Desulfobulbaceae* clone sequences (Table S1, Supporting Information) yielded T-RFs corresponding to the dominant 157 bpT-RF in Fig. 2D, highlighting this taxon as a key toluene degrader under sulfate-reducing conditions. Clones related to Deltaproteobacterial *Desulfovibrio* (GQ898878) and *Polyangiaceae* were exclusively detected in the heavy $^{13}$C-F7 fraction and may also represent primary toluene degraders under sulfidogenic conditions, suggesting some functional redundancy for this role. Alternatively, detection of these taxa in the heavy fraction may result from secondary labeling due to incorporation of $^{13}$C-metabolites. The presence of a small proportion (7%) of *Desulfosporosinus*-related clones in the 'active' $^{13}$C-F7 and a similar proportion (5%) in the unlabeled $^{13}$C-F11 fraction may be due to carryover from the methanogenic inoculum where it presumably dominated. The newly described genus *Youngiibacter* (Lawson *et al.* 2014) was again detected in both fractions from the $^{13}$C-S2S gradient but

more prominently in the light $^{13}$C-F11 fraction; its presence in all clone libraries (including as a singleton) supports interpretation of this Clostridiales member as accessory community member, regardless of electron acceptor. Interestingly, a large proportion (31%) of clones affiliated with Candidate Division OD1 (T-RF 278; Fig. 2D) appeared exclusively in the light $^{13}$C-F10 fraction. The metabolism of OD1 is currently unknown, but it has been detected primarily in anoxic sulphur-replete environments where its involvement in anaerobic methane oxidation has been inferred (Elshahed *et al.* 2005).

Thus, DNA-SIP combined with T-RFLP analysis, cloning, sequencing and *in silico* digestion of density-resolved DNA fractions provided congruent evidence that Firmicutes (mostly *Desulfosporosinus*) dominated the active bacterial community in methanogenic cultures whereas Deltaproteobacteria (primarily *Desulfobulbaceae*) dominated the sulfidogenic bacterial community. This distinct community shift prompted us to search the active DNA fractions for the presence of functional genes characteristic of anaerobic toluene degradation and to affiliate those genes with the dominant taxa.

### *bssA* and *dsrB* functional marker genes detected in SIP gradients

Benzylsuccinate synthase has been identified as the key enzyme for initiating anaerobic toluene degradation (reviewed by Foght 2008). The gene encoding the alpha subunit, *bssA*, is considered a robust functional gene marker for toluene activation under nitrate-, sulfate- and iron-reducing as well as methanogenic conditions (Acosta-González, Rosselló-Móra and Marques 2013). Therefore, to confirm that the dominant phylotypes identified above had the genetic potential for toluene activation, we generated four *bssA* gene clone libraries from the heavy and light fractions for sequencing and phylogenetic analysis. Sequences from 31 clones were translated and aligned with closely related BssA sequences from the NCBI database plus two putative BssA sequences annotated in two draft genomes (*Desulfosporosinus* Tol-M and *Desulfobulbaceae* Tol-SR) assembled from metagenomes of the two $^{13}$C-toluene cultures (Abu Laban *et al.* 2015a, b).

Phylogenetic analysis of 35 translated putative *bssA* sequences (Fig. 4) revealed two closely related clusters similar to clones from contaminated environmental sources and SIP-DNA from a toluene-degrading enrichment culture (GU133298; Winderl *et al.* 2010). The most closely related sequence from a cultivated organism was BssA (WP_006524663; 79% amino-acid similarity) from the fully sequenced *Peptococcaceae* member *Desulfotomaculum gibsoniae*. In addition, a BssA sequence translated from the methanogenic culture metagenome was present in a *Desulfosporosinus* draft genome assembled from this culture (Abu Laban *et al.* 2015a). Thus, although *bssA* has questionable rigor as a phylogenetic marker (Acosta-González, Rosselló-Móra and Marques 2013), all the BssA sequences, whether amplified from methanogenic or sulfidogenic cultures, clearly affiliated with Firmicutes rather than Deltaproteobacteria. This was surprising, as we had expected (from the 16S rRNA gene survey) that *bssA* clones from the sulfidogenic cultures would be affiliated with Deltaproteobacterial *Desulfobulbaceae*; failure to detect these genes in the sulfidogenic *bssA* clones may be due to limited coverage by the small library. Supporting this explanation, a putative *bssA* sequence detected in the $^{13}$C-metagenome of the sulfidogenic culture did affiliate with *Desulfobulbaceae* (EF123667, Winderl, Schaefer and Lueders 2007; and KP009583, Abu Laban *et al.* 2015b). This suggests the presence of at least three *bssA* phylotypes in the $^{13}$C-cultures: two very closely related phylotypes comprising the 35 clones reported here plus a *Peptococcaceae bssA* phylotype from the methanogenic culture metagenome; and one Deltaproteobacterial *bssA* phylotype from the sulfate-reducing metagenome (Fig. 4).

We then questioned why *Desulfosporosinus* T-RF 136, presumably a sulfate-reducer like related cultivated *Desulfosporosinus* spp. (Pester *et al.* 2012), should dominate methanogenic but not sulfate-reducing cultures (Fig. 3A). Translated sequences of 42 cloned *dsrB* genes (a molecular marker for dissimilatory sulfate reduction potential) were compared with known DsrB sequences. Consistent with the 16S rRNA clone library results (Fig. 3A) 80% of translated *dsrB* sequenced from sulfidogenic cultures were closely affiliated with DsrB from *Desulfobulbaceae* (two *Desulfobulbus* clones and *Desulfotalea psychrophila*; Fig. 5); the remaining clones were affiliated with other sulfate-reducing Deltaproteobacteria (*Desulfovibrio* and *Desulfomicrobium*). Notably, none of the SIP-DNA DsrB sequences affiliated with Firmicutes. Indeed, almost all DsrB sequences from the methanogenic cultures (S2M clones) were virtually identical to those from the sulfate-reducing cultures (S2S clones), suggesting that these genes were present in sulfate-reducing Deltaproteobacteria (yellow bars in Fig. 3) that grew fermentatively in methanogenic cultures and reduced sulfate in sulfidogenic cultures.

## DISCUSSION

Toluene has long been used as a model compound for anaerobic degradation of monoaromatic hydrocarbons (e.g. Ficker *et al.* 1999; Beller and Edwards 2000). Its degradation under nitrate-, sulfate- and iron-reducing conditions has been well studied (reviewed by Foght 2008) and new reports have documented degradation under methanogenic conditions (e.g. Fowler *et al.* 2012, 2014; Sun and Cupples 2012; Sun, Sun and Cupples 2014). Toluene is also one of the most labile components of naphtha (Siddique *et al.* 2007), the primary organic carbon source in most OSTP. Gypsum (calcium sulfate) that is added to some oil sands tailings during pond management has provided sulfate to an indigenous sulfidogenic microbial community in some OSTP (Ramos-Padrón *et al.* 2011), whereas in other OSTP either no gypsum was added or the previously added sulfate was depleted (e.g. in Mildred Lake Settling Basin; Holowenko, MacKinnon and Fedorak 2000; Penner and Foght 2010), making methanogenesis possible *in situ*. Because of the significant biogeochemical and physical impacts on tailings caused by indigenous microbial metabolism in OSTP (Fedorak *et al.* 2003; Siddique *et al.* 2014a, b) and the observation of both sulfidogenic (Ramos-Padrón *et al.* 2011) and methanogenic activity (Holowenko, MacKinnon and Fedorak 2000; Penner and Foght 2010), we examined toluene metabolism and community structure under both electron-accepting conditions.

Complete toluene depletion was observed in both methanogenic and sulfidogenic cultures, with the majority of $^{13}$C recovered as $^{13}CO_2 \pm {}^{13}CH_4$ but sufficient label also being incorporated into bacterial and archaeal biomass even during slow growth under methanogenic conditions to permit isolation of isotopically enriched DNA fractions. Although we presume that the most heavily labeled DNA represents the primary (key) toluene degraders, it is possible that some labeling of accessory taxa not directly involved in toluene activation had occurred by the time of sampling (S2M and S2S). However, detection of congruent community structures in both labeled and unlabeled

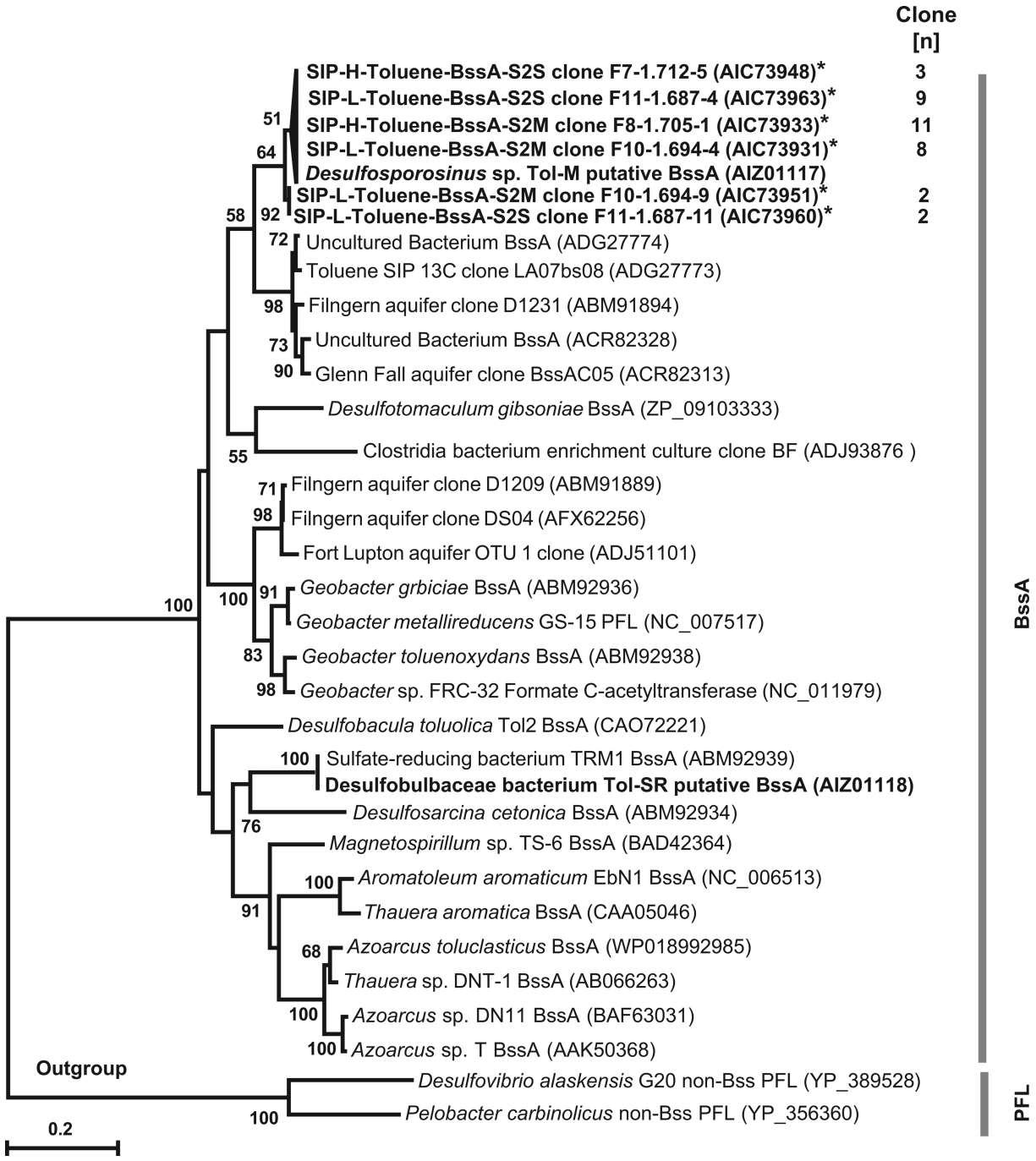


**Figure 4.** Maximum likelihood tree of cloned and translated putative BssA (benzylsuccinate synthase A) sequences amplified from selected DNA-SIP density fractions indicated by asterisks in Fig. 2 (H, heavy fractions; L, light fractions; Table S1, Supporting Information), obtained at time points S2M or S2S. BssA-like sequences (shown in bold; ~258 amino acids) were aligned with those of reference strains and putative BssA sequences annotated in draft genomes assembled from the two culture metagenomes (Abu Laban *et al.* 2015a, b). The dendrogram was rooted with pyruvate formate lyase as an outgroup. The scale bar represents 20% sequence divergence. Asterisks indicate sequences where more than one clone ($n = x$) was obtained and a single representative sequence (accession number) was selected for tree construction.

gradient fractions suggest that the key players have been discerned.

The role of Clostridiales, particularly *Desulfosporosinus* spp., in anaerobic toluene degradation has been highlighted in recent studies of hydrocarbon-contaminated environments and enrichment cultures incubated under sulfate-reducing and/or methanogenic conditions (e.g. Pilloni *et al.* 2011; Sun and Cupples 2012; Fowler *et al.* 2014; Kuppardt *et al.* 2014; Sun, Sun and Cupples 2014). Therefore, it was not surprising that *Desulfosporosinus* T-RF 136 dominated the methanogenic cultures described here and that cloned *bssA* sequences clustered with *bssA* previously detected in Firmicutes (Abu Laban *et al.* 2010; Winderl *et al.* 2010; Acosta-González, Rosselló-Móra and Marques 2013) as there is circumstantial evidence linking this functional marker gene with Clostridiales (e.g. Fowler *et al.* 2012; Sun and Cupples 2012; Kuppardt *et al.* 2014). Indeed, the

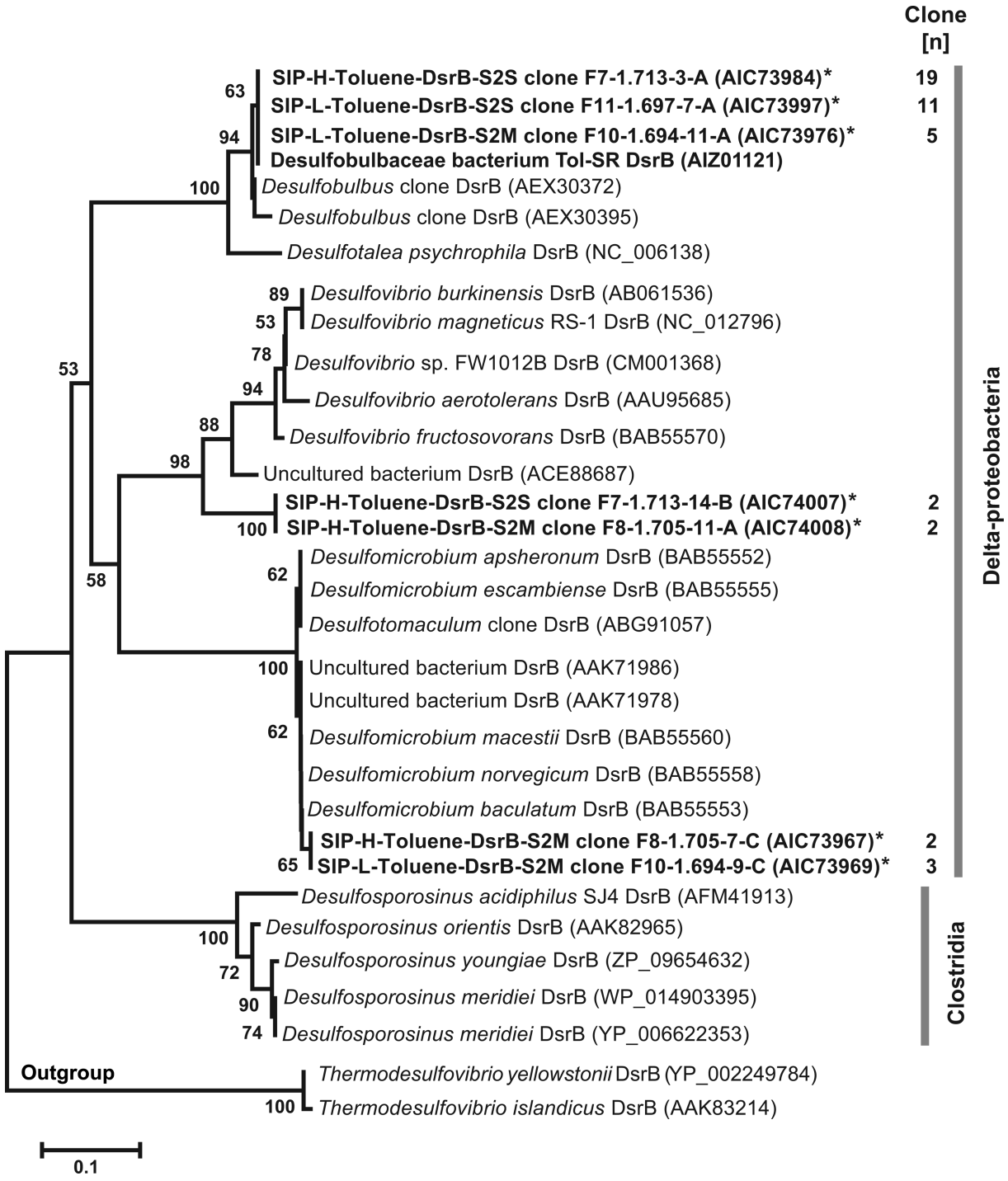


**Figure 5.** Maximum likelihood phylogenetic tree of putative DsrB (dissimilatory sulfite reductase B) sequences (~132 amino acids) amplified from selected density fractions (indicated by asterisks in Fig. 2) obtained at time points S2M or S2S. DsrB-like sequences (shown in bold) were aligned with DsrB reference sequences and a DsrB sequence assembled from the sulfidogenic culture metagenome (Abu Laban *et al.* 2015b). The dendrogram was rooted with *Thermodesulfovibrio* DsrB as an outgroup. The scale bar represents 10% sequence divergence. Asterisks indicate sequences where more than one clone ($n = x$) was obtained and a single representative sequence (accession number) was selected for tree construction.

$^{13}$C-DNA *bssA* gene cluster detected here included a putative *bssA* annotated in a *Desulfosporosinus* draft genome assembled using metagenomic DNA from the same culture (Abu Laban *et al.* 2015a). Rather, it was the rapid emergence of *Desulfobulbaceae* T-RF 157 bp under sulfate-reducing conditions that was unexpected, since previously described *Desulfosporosinus* spp. have been demonstrated or inferred to be sulfate reducers (Robertson, Franzmann and Mee 2000; Pester *et al.* 2012) and were expected in the current study to persist as the dominant toluene-degrading taxon under both electron-accepting conditions.

This shift in dominant taxa can be explained at least in part by the apparent absence of a canonical *dsrB* gene associated

with *Desulfosporosinus* T-RF 136 and consequently its inferred inability to use sulfate as an electron acceptor. The absence of *Desulfosporosinus*-affiliated *dsrB* in the clones was confirmed by analyzing the draft genome of *Desulfosporosinus* Tol-M (corresponding to T-RF 136) in which neither *dsrB* nor *dsrMKJOP* genes used in sulfite reduction were detected (Abu Laban *et al.* 2015a). Our inability, despite numerous attempts, to cultivate *Desulfosporosinus* T-RF 136 in sulfate-reducing medium with various carbon sources provides additional, albeit circumstantial, evidence that this *Desulfosporosinus* is incapable of sulfate reduction: it did not grow with sulfate as the terminal electron acceptor and in fermentative medium persisted only with syntrophic partners (N. Abu Laban *et al.*, unpublished results). Therefore, this uncultivated *Desulfosporosinus* T-RF 136 may be an obligate fermentative syntroph, in contrast to the cultivated *Desulfosporosinus* sp. Y5 that degrades toluene with arsenate reduction and furthermore uses various inorganic electron acceptors (sulfate, nitrate, thiosulfate and ferric iron; Liu *et al.* 2004). This apparent physiological difference is striking since phylogenetic analysis (Fig. S3, Supporting Information) placed 10 cloned 16S rRNA genes from the methanogenic heavy fraction in a sister clade to *Desulfosporosinus* sp. Y5. Although loss of *dsrB* has not previously been reported for *Desulfosporosinus* spp., Imachi *et al.* (2006) proposed that *Desulfotomaculum* subcluster Ih (a related clade of *Peptococcaceae*) had lost the ability to respire with sulfate and became strictly fermentative in syntrophic association with methanogens. In this study, we propose that *Desulfosporosinus* T-RF 136 is a key toluene degrader by virtue of its *bssA* genes, and outcompetes *Desulfobulbaceae* T-RF 157 under methanogenic conditions, but conversely is unable to compete with the sulfate-reducing *Desulfobulbaceae* T-RF 157 under sulfidogenic conditions.

The observed dominance of *Desulfobulbaceae* affiliated with *Desulfocapsa* (Fig. S3, Supporting Information) in the sulfidogenic culture 16S rRNA gene library (Fig. 3A) and also in the *dsrB* clone library (80% of clones were affiliated with *dsrB* in *Desulfobulbus* spp.; Fig. 5) is plausible since members of this Deltaproteobacterial family have been associated with monoaromatic hydrocarbon degradation under both sulfidogenic (e.g. Winderl *et al.* 2010; Pilloni *et al.* 2011; Sun and Cupples 2012; Kuppardt *et al.* 2014) and methanogenic conditions (Sun, Sun and Cupples 2014). The same *dsrB* gene was detected in the *Desulfobulbaceae* Tol-SR draft genome assembled from the sulfidogenic $^{13}$C-toluene culture metagenome (Abu Laban *et al.* 2015b) and we also detected *dsrB* genes affiliated with other Deltaproteobacteria (*Desulfomicrobium* and *Desulfovibrio* spp.) (Fig. 5), supporting the 16S rRNA gene clone library results (Fig. 3A).

Curiously, although *Desulfobulbaceae* T-RF 157 dominated the active sulfidogenic SIP fractions, no Deltaproteobacterial *bssA* sequences were detected in the clone libraries. There are at least two possible explanations for this observation. First, *Desulfobulbaceae* T-RF 157 may harbor divergent *bssA* gene(s) not amplified by the 7772f/8546r primers; second, a library of only 39 clones may be insufficient to detect the full diversity of *bssA* gene phylotypes. Since we detected a putative *bssA* gene in the *Desulfobulbaceae* Tol-SR draft genome without amplification (Abu Laban *et al.* 2015b), the first explanation invoking primer bias seems more likely. Thus, this *Desulfobulbaceae* genome apparently harbors a non-canonical *bssA* that differs from the suite of known *Desulfosporosinus bssA* sequences, as shown by its distant placement in Fig. 4. In fact, the existence of divergent *bssA* phylotypes (and variants of the related alkylsuccinate synthase alpha subunit gene, *assA*) has been highlighted recently (Acosta-González, Rosselló-Móra and Marques 2013; von Netzer *et al.* 2013), suggesting that less stringent *bssA* primers are required to reveal this gene's full diversity.

We therefore propose a working hypothesis in which syntrophic toluene metabolism under methanogenic conditions comprises activation of toluene by *Desulfosporosinus* T-RF 136 in syntrophy with *Methanosaeta* and *Methanoculleus*. It is possible that incomplete oxidation of toluene to acetate by *Desulfosporosinus* T-RF 136 supports acetoclastic *Methanosaeta* (T-RF 280 bp) and ancillary fermenters such as Proteobacteria (*Youngiibacter* T-RF 515 and Epsilonproteobacterium T-RF 462) and Spirochaetes detected in clone libraries, whereas the metabolic products $H_2 + CO_2$ may support a smaller population of hydrogenotrophic *Methanoculleus* (T-RF 181 bp). Using SIP-DNA, Sun, Sun and Cupples (2014) similarly implicated *Desulfosporosinus* as the key toluene consumer in a methanogenic soil culture, but found different secondary fermenters (*Peptostreptococcaceae* and *Pseudonocardia*) apparently supporting several genera of *Methanomicrobia*. Under sulfidogenic conditions, we infer complete toluene oxidation by *Desulfobulbaceae* (perhaps aided by other Deltaproteobacteria), based on the recovery of a high proportion of electrons from toluene as sulfide, and $^{13}CO_2$ production near the theoretical maximum from $^{13}CO_6$-toluene. However, this working model requires additional biochemical evidence to support or refute the proposed role(s) of these taxa.

### Significance to OSTP and other hydrocarbon-impacted anaerobic environments

It is significant that toluene degradation and sulfide production began very quickly after sulfate addition and that the dominant Bacteria quickly shifted to sulfate-reducing taxa. That is, the potential for sulfidogenic toluene metabolism was retained by the enrichment cultures despite the inoculum source (the OSTP) being predominantly methanogenic and despite these tailings undergoing two passages under methanogenic conditions in the laboratory prior to use. Pyrosequencing previously revealed *Desulfosporosinus* and *Desulfobulbaceae* (*Desulfocapsa* and *Desulfobulbus* spp.) plus other taxa detected in the current study, to be prevalent genera in two OSTP, coal bed seams and conventional oil fields (An *et al.* 2013), suggesting their importance in different hydrocarbon-impacted anaerobic environments that may experience changes in electron acceptor availability. Reports of both sulfidogenic and methanogenic activity in OSTP (Penner and Foght 2010; Ramos-Padrón *et al.* 2011) support the conclusions of the current study, namely that oil sands tailings microbial communities are metabolically flexible, likely have functional redundancy, and can rapidly adjust to toluene degradation under different electron-accepting conditions. This information is important for effective management of OSTP and predicting microbial impacts in the form of greenhouse gas emissions and/or hydrogen sulfide production during operation or reclamation of OSTP. It also speaks to the potential for resiliency and community adaptation during future reclamation of oil sands tailings.

## SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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