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Molecular characterisation of two anaerobic phenol-degrading enrichment cultures

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ABSTRACT

The microbial composition of two phenol-degrading enrichment cultures was characterised using molecular techniques and by analysing their degradation capacity. The cultures originate from two different anaerobic bioreactors treating organic household waste at mesophilic and thermophilic temperature. The results showed that two unique community structures had developed, with the ability to partially or completely degrade phenol and 4-hydroxybenzoate. These compounds were degraded at temperature up to 48 °C in both cultures. Phylogenetic analysis confirmed the presence of one conceivable phenol degrader in each culture; one affiliated to subcluster Ih in the phylum *Desulfotomaculum* and the other to the family *Syntrophorhabdaceae* in the phylum delta *Proteobacteria*. This study confirms the importance of both these clusters for bacteria degrading aromatic compounds, especially phenols and phthalate isomers, under methanogenic conditions.

1. Introduction

Phenols are widespread compounds that can have a natural or anthropogenic source. They can be produced from biodegradation of naturally occurring aromatic amino acids and aromatic polymers, *e.g.* humic acids, lignins and tannins in plant material, or from degradation of different xenobiotic compounds, such as pesticides, or during various industrial manufacturing (van Schie and Young, 1998; Fang et al., 2006). Phenolic compounds can also be produced during different pre-treatments of lignocellulosic material. Pre-treatments are used to increase the efficiency during production of biofuels such as biogas and bioethanol (Klinke et al., 2004; Chen et al., 2006). Consequently, phenols are present in different industrial wastewaters and in sewage sludge (Angelidaki et al., 2000; Veeresh et al., 2005; Khardenavis et al., 2008). They are also found in digestate from anaerobic bioreactors degrading different organic materials, such as manure, municipal solid waste and plant materials (Levén and Schnürer, 2005; Levén et al., 2006).

Many phenols are toxic at high concentrations to different groups of microorganisms, including organisms in the biogas process (Dyreborg and Arvin, 1995; Varel and Miller, 2001; Olguin-Lora et al., 2003; Levén et al., 2006; Hernandez and Edyvean, 2008). Phenols have also been shown to decrease the activities of microorganisms in soil, which can be a problem when digestate is applied as a fertilizer (Levén et al., 2006). The concentrations of phenols in the biogas process and the digestate, and thus their inhibitory effects, have been shown to be related to both the input material and operational parameters, *i.e.* process temperature. One study has shown that anaerobic bioreactors with a high input of swine manure generally have a higher content of phenols in the digestate compared with bioreactors receiving less manure (Levén et al., 2006). Phenolic compounds have previously been identified in swine manure (Wu et al., 1999). The degradation of phenols has been shown to be strongly influenced by temperature, with a higher degradation capacity at mesophilic temperature compared with thermophilic (Levén and Schnürer, 2005; Levén et al., 2006).

Under methanogenic conditions, the phenol mineralisation process is complex and can proceed through different pathways requiring a consortium of various microorganisms. So far, two possible pathways for mineralisation of phenol into methane have been reported; via 4-hydroxybenzoate into benzoyl-CoA or via caproate into acetate. The degradation of phenol via benzoate is well documented and has been shown to occur in several methanogenic consortia (Knoll and Winter, 1989; Kobayashi et al., 1989; Sharak Genthner et al., 1991; Karlsson et al., 2000; Fang et al., 2004, 2006; Chen et al., 2008, 2009). However, only a few of these have been characterised concerning microbial populations (Zhang et al., 2005; Fang et al., 2006; Chen et al., 2008, 2009). Furthermore, only three phenol-degrading bacteria have been isolated from methanogenic environments; *Sedimentibacter hydroxybenzoicum*

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(previously known as *Clostridium hydroxybenzoicum*) (Zhang et al., 1994), *Cryptanaerobacter phenolicus* (also known as 'Strain 7') (Juteau et al., 2005) and *Syntrophorhabdus aromaticivorans* (Qiu et al., 2008) The limited number of isolates is most likely due to the fact that isolation of a phenol degrader in pure culture under methanogenic conditions can be obstructed by its use of only a limited substrate range, the need for syntrophic relationships and unknown growth factors (Schink, 1997; Karlsson et al., 2000; Juteau et al., 2005;Qiu et al., 2008).

The aim of this study was to supply further information on microorganisms involved in methanogenic phenol degradation. The microbial composition of two phenol-degrading enrichment cultures was characterised using PCR primers specific for *Bacteria* or *Archaea*, followed by cloning and sequence analysis. The phenol-degrading capacity of the cultures was also investigated in anaerobic batch experiments. The cultures originated from two anaerobic laboratory-scale bioreactors treating organic household waste at 37 °C or 55 °C. These bioreactors have previously shown different phenol-degrading capacity (Levén and Schnürer, 2005).

2. Materials and methods

2.1. Enrichment procedure

Serial dilutions were made of the biological active material from two semi-continuous stirred 45 L bioreactors operated at 37 °C (mesophilic) or 55 °C (thermophilic) temperature (Levén and Schnürer, 2005) by successive transfers of 2 mL of the initial culture to 18 mL of growth medium in 118 mL cultivation bottles. The preparation of culture medium was carried out as described previously by Levén and Schnürer (2005) except that cysteine was replaced with sodium dithionite (19 mg/L). Phenol was added by syringe from sterile stock solutions to a final concentration of 0.5 mM. The cultures were incubated at 37 °C in darkness and without shaking. The reason for incubating the culture originating from the thermophilic bioreactor at mesophilic temperature was that no or limited phenol degradation occurred in the bioreactor at 55 °C, while efficient degradation was observed at 37 °C (Levén and Schnürer, 2005). The most dilute culture in which phenol degradation occurred was used to initiate a new set of diluted cultures in a similar manner to that described above. This enrichment procedure was repeated six times. The enrichment cultures obtained from the mesophilic and thermophilic bioreactors were denoted MR and TR, respectively.

2.2. Microscopic examination

A Zeiss Axioscope equipped with a mercury lamp for epifluorescence was used to perform phase contrast microscopy and study the morphology of bacteria and methanogens in the enriched cultures. With epifluorescence microscopy most methanogens are easily distinguished from bacteria, due to presence of coenzyme F_{420} (Cheeseman et al., 1972).

2.3. Anaerobic degradation experiment

Phenol, *p*-cresol, 4-hydroxybenzoate (4-OHBa) and phthalate were transferred from sterile stock solutions to duplicate bottles with cultivation medium, prepared as described in section 2.1, to obtain a final concentration of 0.5 mM. The medium was then inoculated with 2 mL of the final enrichment culture. The cultures were incubated in the dark without shaking at 37 and 48 °C and, for the TR-culture also at 55 °C. The degradation of the added compounds and the production of intermediates such as benzoate were measured by HPLC analysis according to Levén and Schnürer (2005). Production of acetate and other fatty acids was investigated by HPLC analysis as described by Levén et al. (2007).

2.4. DNA extraction

The isolation of DNA from the two enrichment cultures was performed according to the manufacturer's protocol instructions for the Dneasy Tissue KitTM (Qiagen, Valenca, CA, USA).

2.5. PCR amplification, cloning and sequencing

PCR amplification of bacterial and archaeal 16S rDNA was carried out as described by Levén et al. (2007). Primers EC9-26f and 926r or A571F and UA1204R were used producing fragments with the length of about 900 bp and 650 bp respectively (Baker et al., 2003; Jernberg and Jansson, 2002; Levén et al., 2007). Bands were excised from agarose gel (1%, Ultrapure, MB Grade, USB Corporation, OH, USA) after detection and size control. DNA was purified from gel slices using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany).

Clone libraries were constructed for *Bacteria* and *Archaea* present in the two enrichment cultures by cloning PCR products from each culture. The purified amplicons were first ligated into the pCR® 4-TOPO® vector from a TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA). Ligations were then transformed into DH5α™-T1® competent *Escherichia coli* (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Randomly selected colonies were purified prior to isolation of plasmid DNA (QIAprep® Spin Miniprep Kit, Qiagen, Hilden, Germany). Sequencing was performed by Macrogen (Seoul, Korea) using the plasmid-specific primers T3 and T7. The mesophilic and thermophilic bacterial clones were denoted P and TP, respectively, whereas the mesophilic and thermophilic archaeal clones were denoted PA and TPA, respectively.

The clones were screened by Amplified Ribosomal DNA Restriction Analysis (ARDRA). After transformation and purification of plasmids, the presence of insert was verified by reamplification with bacterial and archaeal primers. The same primers as in the PCR reactions preceding the cloning were used, but without the addition of Bovine Serum Albumin (BSA). The amplicons of the 16S rDNA were digested overnight at 37 °C with 3 units of HaeIII and HhaI (New England BioLabs Inc, USA). The resulting fragments were analysed by 2% agarose (Ultrapure, MB Grade, USB Corporation, OH, USA) gel electrophoresis and compared using the GelCompar II version 4.5 software (Applied Maths, Belgium). Clones from the cultures with matching band pattern in ARDRA and sequences with identity >98% were considered to be the same operational taxonomic unit (OTU). Library coverage (C), a measure of the diversity captured, was calculated as $C = 1 - (n/N)$, where n is the number of different OTU types from a clone library that are encountered only once and N is the total number of clones analysed.

2.6. Sequence analysis

Derived nucleotide sequences of 16S rDNA were compared with sequences in both the GenBank (NCBI) database and Ribosomal Database Project (RDP; URL: http://rdp.cme.msu.edu/; Cole et al., 2007), followed by investigation of chimeric properties using CHIMERA_CHECK (URL: http://35.8.164.52/cgis/chimera.cgi?su=SSU). Phylogenetic analyses were conducted using software MEGA version 4 (Tamura et al., 2007). The sequences were aligned with nucleotide sequences from the database using the CLUSTAL W software and the Jukes and Cantor correction (Jukes and Cantor, 1969) was used for distance matrix analyses. Finally, a phylogenetic tree was constructed using the neighbour-joining method

(Saitou and Nei, 1987). The tree topology was evaluated by bootstrap analysis of 1000 replicates.

2.7. Accession numbers

The GenBank accession numbers for the partial 16S rDNA sequences obtained in this study are GQ377457–GQ377459 for *Archaea* and GQ377460–GQ377476 for *Bacteria*.

3. Results

3.1. Impact of temperature on anaerobic degradation of phenols

The TR enrichment culture from the bioreactor run at thermophilic process temperature (55 °C) degraded phenol at 48 °C and below, but no degradation occurred at 55 °C (Fig. 1A). During enrichment of this culture the ability to degrade benzoate was lost, which was manifested as an accumulation of benzoate. The same pattern and effect of temperature was seen for 4-hydroxybenzoate *i.e.* degradation occurred below 48 °C and benzoate was accumulated. No transformation was observed for *p*-cresol and phthalate at any temperature. The mesophilic phenol (MR) enrichment culture, on the other hand, degraded phenol to methane at temperatures at both 37 and 48 °C (Fig. 1B). The only intermediate that was observed was benzoate and it was further degraded. Complete degradation was also seen for 4-hydroxybenzoate at 48 °C and below. As for the TR culture, *p*-cresol and phthalate were not degraded.

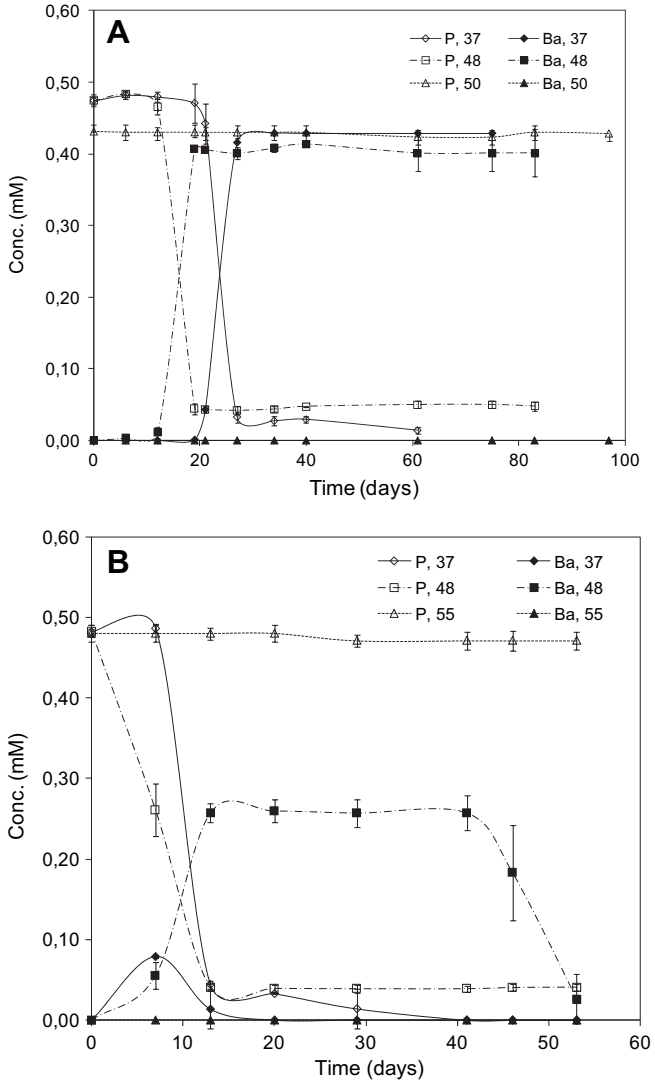

Fig. 1. Anaerobic degradation of phenol via benzoate in TR (A) and MR (B) enrichment cultures at different incubation temperatures.

3.2. Enrichment cultures

Microscopic examination of the MR culture showed the presence of at least four dominant morphologically different bacteria and one methanogen, with fluorescent capacity and an irregular coccus shape. The most dominant bacterial shape was a rod with blunted ends. The three additional dominant bacteria were rod-shaped, one with pointed ends. In the TR culture a higher variety of bacteria was found. One of the most dominant bacteria was rod-shaped, but also methanogens with fluorescent capacity were seen.

3.3. Molecular characterisation of the enrichment cultures

For the bacterial clone libraries, 114 clones were analysed from the MR culture and 153 from the TR culture. In total, 6 and 11 unique sequences types or operational taxonomy units (OTUs) were identified in the MR and TR clone libraries, respectively (Tables 1 and 2). The most dominant OTU, 22P, represented 50% of the MR clone library. Its sequence clustered together with anaerobic environmental clones and one isolate, *S. aromaticivorans*, belonging to the novel family *Syntrophorhabdaceae* in the phylum delta *Proteobacteria* (previously known as delta *Proteobacteria* group TA; Fig. 2). One OTU (2P, 19%) affiliated with the phylum *Thermotogae*, one (8P; 14%) with *Bacteroidetes* and one (53P, 3%) with *Synergistetes*. In addition, two OTUs (34P and 23P, 14%) had unclear affiliation.

The thermophilic enrichment culture was dominated by bacteria affiliated with the phyla *Synergistetes* (27%) and *Bacteroidetes* (21%). The OTUs 8TP and 12 TP were closely related, with 99% sequence identity, to *Anaerobaculum mobile* and *Bacteroidetes* sp. 22C, respectively. Furthermore, five OTUs belonged to the phylum *Firmicutes*, class *Clostridia*, and were dispersed between different orders, *Clostridiales* (11TP, 18%), *Desulfotomaculum* subclass Ih (15TB, 5%; Fig. 3), *Thermobacterales* (32TP, 5%) and *Thermoanaerobacterales* (44TP, 2%). Other OTUs with a low abundance in the clone library affiliated with the phylum *Thermotogae* (21TP and 29TP, 9%), epsilon *Proteobacteria* (TB38, 6%), *Spirochaetes* (83TP, 4%) and *Chloroflexi* (80TP, 1%).

In the archaeal clone libraries, 38 and 50 clones from the MR and TR cultures, respectively, were screened. In the MR culture, one OTU was found to correspond to *Methanoculleus palmaeoli* (99% sequence identity). Two methanogens were identified among the clones from the TR culture and these showed 98–99% sequence identity to *Methanosarcina mazei* and *M. palmaeoli*. They represented 43% and 57% of the archaeal clones library from the TR

Table 1
Affiliation and distribution of bacteria 16S rRNA gene sequences analysed from the mesophilic (P) enrichment culture.

Putative division	OTU	% of total clones	Closest sequence/ isolate	Assession no.	Sequence identity%
Delta Proteobacteria	22P	50	*Syntrophorhabdus aromaticivorans*	AB212873	95
Thermotogae	2P	19	Thermotogales bacterium M79	AY692052	99
Bacteroidetes	8P	14	Bacteroides sp.22C	AY554420	96
Synergistetes	53P	3	*Aminobacterium colombiense*	AF069287	95
Unknown	34P	11	PS7	DQ984664	99
Unknown	23P	3	ORS40C_e04	EF393052	95

Table 2
Affiliation and distribution of bacteria 16S rRNA gene sequences analysed from the thermophilic (TP) enrichment cultures.

Putative division	OTU	% of total clones	Closest sequence/isolate	Assession no.	Sequence identity%
Synergistetes	8TP	27	*Anaerobaculum mobile*	AJ243189	99
Bacteroidetes	12TP	21	Bacteroides sp.22C	AY554420	99
Firmicutes, Clostridia	11TP	18	*Clostridium sartagoformum*	Y18175	99
	15TB	5	*Crypanaerobacter phenolicus*	AY327251	93
	32TP	5	*Coprothermobacter proteolyticus*	X69335	99
	44TP	2	*Tepidanaerobacter syntrophicus*	AB106353	100
Thermotogae	29TP	6	Thermotogales bacterium M79	AY692052	99
	21TP	3	Thermotogae bacterium SHBZ1050	EU639347	99
Epsilon Proteobacteria	38TB	6	*Wollinella succinogenes*	M88159	99
Spirochaetes	83TP	4	E3	AY426468	99
Chloroflexi	80TP	1	SJA-68	AJ009475	96

culture, respectively. The calculated values of library coverage (0.93–0.97) showed that the clone libraries gave a good picture of the microbial community (*Archaea* and *Bacteria*) in both enrichment cultures.

4. Discussion

The two phenol-degrading enrichment cultures, MR and TR culture, analysed in this study were obtained from two anaerobic bioreactors, one operating at mesophilic temperature (37 °C) and one at thermophilic temperature (55 °C). The culture originating from the lower process temperature (MR culture) was capable of complete phenol and 4-hydroxybenzoate degradation, while the transformation in the TR culture ended in benzoate (Fig. 1A and B). One possible explanation for the lack of benzoate degradation in the TR culture is that the benzoate-degrading organisms were lost during the enrichment procedure, possibly due to lowering of the original process temperature from 55 to 37 °C. However, in the

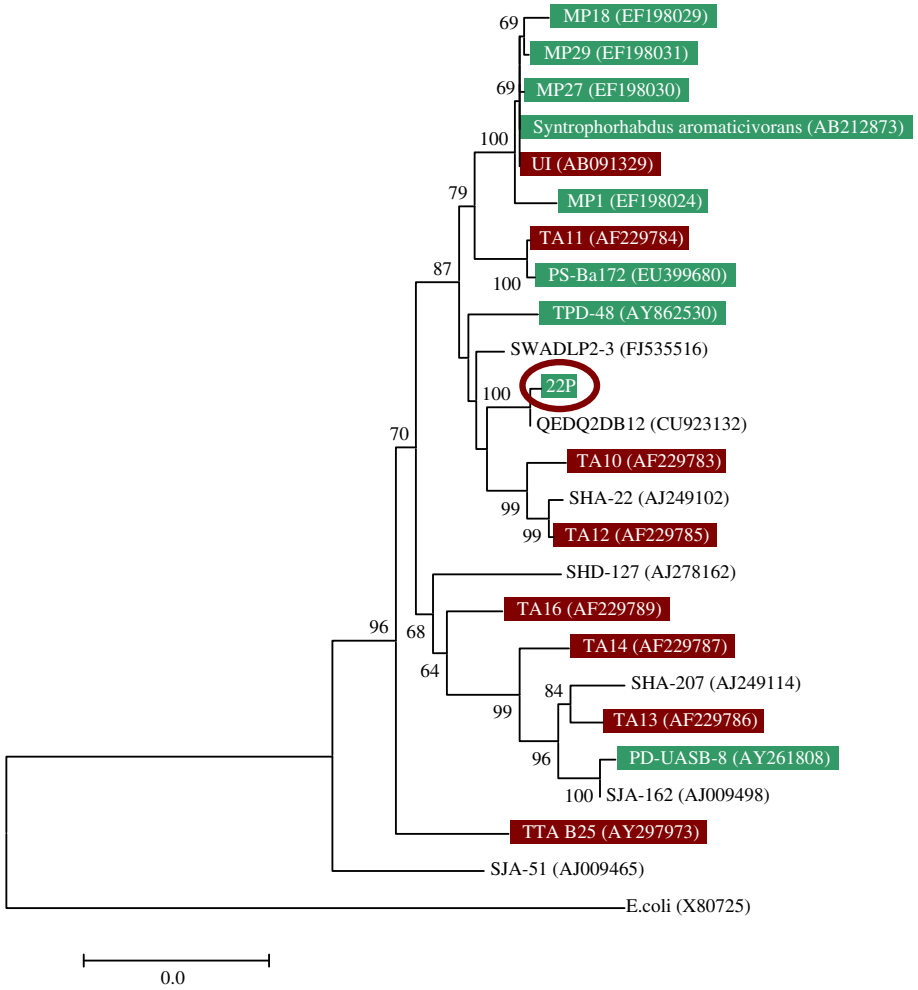

Fig. 2. Phylogenetic relationships in *Syntrophorhabdaceae* based on neighbour-joining analysis of partial 16S rDNA sequences. Bootstrap values are shown for nodes that had >50% support in a bootstrap analysis of 1000 replicates. The accession number is given in brackets and encircled is the probable phenol-degrader. Marked with green are sequences from other known phenol degrading co-cultures or isolates, and red are bacteria capable of degrading different phthalate isomers.

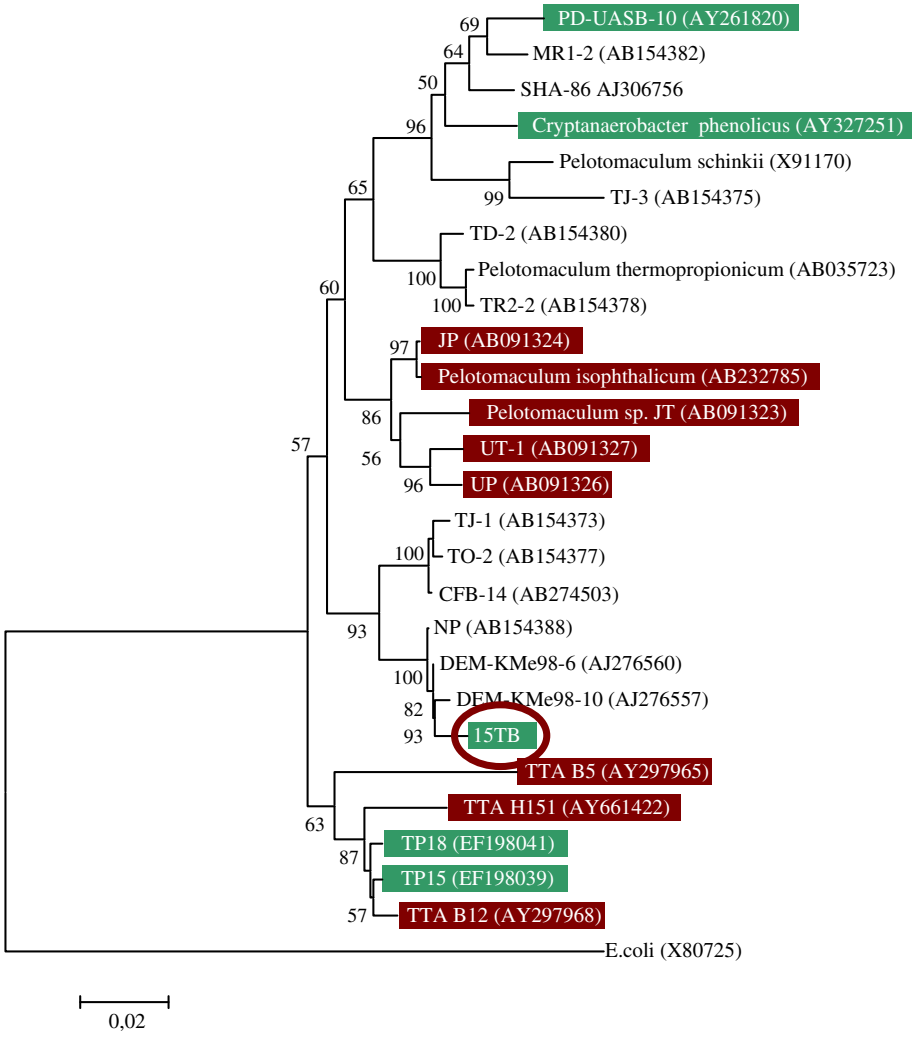

Fig. 3. Phylogenetic relationships in *Desulfomaculum* subcluser Ih based on neighbour-joining analysis of partial 16S rDNA sequences. Bootstrap values are shown for nodes that had >50% support in a bootstrap analysis of 1000 replicates. The accession number is given in brackets and encircled is the probable phenol-degrader. Marked with green are sequences from other known phenol degrading co-cultures or isolates, and red are bacteria capable of degrading different phthalate isomers.

thermophilic bioreactor used as inoculum for the enrichment, benzoate has been shown to be completely broken down to methane and carbon dioxide at 37–55 °C (Levén and Schnürer, 2005). Confirming was that no clones affiliated with any known benzoate degrader in the phylogenetic analysis of the TR clone library. Alternatively, the lack of benzoate degradation could have been caused by low activity of the methanogenic population. Complete degradation of benzoate in a methanogenic environment can only proceed in a syntrophic consortium including a methanogen (Hopkins et al., 1995). However, both with microscopic examination and sequence analysis presence of methanogens were confirmed (Table 2).

The impact of temperature on the degradation of phenol was the same for both enrichment cultures, *i.e.* no 4-hydroxybenzoate or phenol degradation occurred above 48 °C. This temperature effect is in agreement with previous studies on the anaerobic bioreactors used as inocula for the MR and TR culture in the present study (Levén and Schnürer, 2005). Furthermore, a lowering of process temperature below 48 °C was reported to trigger the degradation of phenols in batch cultures started with material from several large-scale anaerobic bioreactors run at thermophilic temperature (Levén et al., 2006). The explanation for this activation is unclear but it may be due to the impact of temperature on key enzymes, degradation routes and/or microbial populations. Phenol degradation has been shown to occur at thermophilic temperature (Karlsson et al., 1999; Fang et al., 2006; Levén et al., 2006), but possibly using a different degradation route, via caproate rather than via benzoate as at mesophilic temperature (Fang et al., 2006). Furthermore, temperature has been shown to affect the composition of the microbial populations in anaerobic bioreactors (Sekiguchi et al., 1998; Pender et al., 2004; Hernon et al., 2006; Levén et al., 2007).

Only a few reports to date describe the characterisation of syntrophic phenol-degrading consortia. Chen et al. (2008) identified two different predominant bacteria populations in a mesophilic and a thermophilic phenol-degrading methanogenic consortium enriched from a wastewater treatment plant. These bacteria affiliated with members of the family *Syntrophorhabdaceae* in the phylum delta *Proteobacteria* and subcluster Ih in the phylum *Desulfotomaculum*, respectively (Figs. 2 and 3). Three other previous studies characterising phenol-degrading microbial communities were performed with materials from two upflow anaerobic sludge blankets (UASB). These reactors were seeded with the same inoculum and fed with phenol as the only carbon source, but run at different process temperatures (Fang et al., 2004, 2006; Zhang et al., 2005). Although phenol was mineralised via caproate at the thermophilic temperature, no phenol degrader could be clearly

identified (Fang et al., 2006). However, one clone (TPD-48) at low abundance affiliated with members of the *Syntrophorhabdaceae*. In the UASB reactor operating at ambient temperature, phenol was degraded via benzoate, possibly by a bacteria represented by the clone PD-UASB-10 with 97% sequence identity to *C. phenolicus* (Fang et al., 2004; Zhang et al., 2005). In addition, the most predominant bacterial population in a full-scale anaerobic fluidised bed reactor treating phenolic wastewater at mesophilic temperature has been shown to belong to the *Syntrophorhabdaceae* (Chen et al., 2009).

Previous investigations suggest two clusters, *Syntrophorhabdaceae* and subcluster Ih, *Desulfotomaculum*, as two important groups of bacteria capable of degrading aromatic compounds such as phenol, benzoate, 4-hydroxybenzoate and phthalate isomers under methanogenic conditions (Qiu et al., 2004; Chen et al., 2008, 2009). Our results provide further support for this suggestion. Based on the sequence analysis in our study, two different bacteria are suggested to carry out the phenol degradation in the enrichment cultures. In MR culture, the possible phenol degrader (22P) is assigned to the *Syntrophorhabdaceae* in delta *Proteobacteria*, with a 95% sequence identity to *S. aromaticivorans* (Table 1 and Fig. 2). This clone dominated the clone library from the MR culture. The probable phenol degrader in the TR culture (15TB) affiliated with members of *Desulfotomaculum*, subcluster Ih, showing 93% identity to *C. phenolicus* (Table 2 and Fig. 3). The low abundance of this OTU in the thermophilic clone library (Table 2) may be an effect of slow growth rate. The conversion of phenol into benzoate is thermodynamically unfavourable and the reaction does not generate any energy (Li et al., 2000). Identification of this organism as the phenol degrader is supported by the fact that its degradation pattern is similar to that of *C. phenolicus*, which can only convert phenol into benzoate in the presence of unknown growth factor(s) (Juteau et al., 2005; Qiu et al., 2008). In comparison, *S. aromaticivorans* is known to transform phenol to acetate and methane in cooperation with a methanogen. Thus, the different degradation patterns observed in our two enrichment cultures conform to what would be expected from the suggested phenol-degrading organisms.

The dominance of bacteria with a close relationship (97–99%) to *A. mobile* and uncultured *Bacteroides* sp. 22 in the TR culture (Table 2) can probably be explained by the addition of yeast extract to the culture. None of these bacteria have shown the ability to degrade phenol, but they can grow with yeast extract as sole carbon and energy source (Menes and Muxí, 2002; Chen and Dong, 2005). However, addition of yeast extract was essential for the degradation of phenol to proceed. The degradation of yeast extract may produce growth factors, which can be essential for the phenol degrader.

One interesting question concerning the MR culture is the organisms that perform acetate degradation. Cleavage of the aromatic ring followed by oxidation of the fatty acid produces acetic acid. As this acid was not accumulated in the culture, it has to be degraded. Acetotrophic methanogens or acetate-oxidising bacteria in cooperation with hydrogenotrophic methanogens have the ability to degrade acetate. However, neither methanogens with acetotrophic activity nor any clones closely related to known syntrophic acetate-oxidising bacteria *e.g.* strain AOR, *Clostridium ultunense*, *Thermacetogenium phaeum* nor *Thermotoga lettingae* (Hattori, 2008) were present in the MR enrichment culture (Table 1). In our mesophilic clone library, the second most abundant clone (2P) affiliated with members of the *Thermotogae*, but with only 80% sequence identity to *T. lettingae*. Thus, the organisms that performed acetate degradation remain unidentified.

To conclude, clone sequences found in our enrichment cultures differed, suggesting development of unique phenol-degrading microbial community structures, most likely as an effect of different inoculum origin and process temperatures. Phylogenetic analysis confirmed the presence of one conceivable phenol degrader in each culture. The two probable phenol degraders identified belonged to the *Syntrophorhabdaceae* and subcluster Ih, *Desulfotomaculum*, respectively. This, together with previous findings, shows that these two clusters can be used as indicators of anaerobic degradation of phenol and phthalate under methanogenic conditions. Furthermore, phenol degradation is affected by temperature, so in order to increase the phenol degradation and thereby decrease the inhibitory effects of material rich in phenolic compounds, mesophilic process temperatures are recommended.

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