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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