Microbial characteristics of a methanogenic phenol-degrading sludge

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Abstract Microbial properties of a methanogenic granular phenol-degrading sludge were characterized using the 16S rRNA/DNA-based techniques, including polymerase chain reaction (PCR) amplification, cloning, DNA sequencing, and fluorescence *in situ* hybridization (FISH). The sludge was sampled from an upflow anaerobic sludge blanket reactor, which removed 98% of phenol (up to 1260 mg/l) in wastewater at 26°C with 12 hours of hydraulic retention. Based on DNA analysis, the *Eubacteria* in the sludge was composed of 13 operational taxonomy units (OTUs). Two OTUs, one resembling *Clostridium* and the other remotely resembling *Desulfotomaculum*, were likely responsible for the conversion of phenol to benzoate, which was further degraded by five *Syntrophus*-resembling OTUs to acetate and H_2/CO_2; methanogens lastly converted acetate and H_2/CO_2 into methane. The role of six remaining OTUs remains unclear. Overall, the sludge was composed of 26 ± 6% *Eubacteria* and 74 ± 9% methanogens, of which 54 ± 6% were acetotrophic *Methanosaetaceae*, 14 ± 3% and 3 ± 2% were hydrogenotrophic *Methanomicrobiales* and *Methanobacteriaceae*, respectively.

Keywords Benzoate; degradation; FISH; methanogenic; phenol; 16S rDNA

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Introduction

Phenol is a toxic pollutant common in the wastewater of polymeric resin production, coal gasification, oil refining, coking, etc. (Kirk, 1978). Phenol in wastewater may be removed either by physico-chemical means (Larson and Chuang, 1994) or by biological means (Fang *et al.*, 1996). It can be degraded aerobically by a number of bacteria, including *Trichosporon* sp. (Santos *et al.*, 2001), and *Bacillus thermoglucosidasius* (Duffner *et al.*, 2000). It can also be degraded anaerobically under denitrifying (Shinoda *et al.*, 2000), sulfate reducing (Boopathy, 1997) and methanogenic (Fang *et al.*, 1996) conditions. However, only a few anaerobic phenol-degrading bacteria have been isolated to date. Most denitrifying bacteria degrading aromatic chemicals that have been isolated are *Thauera* (Anders *et al.*, 1995) and *Azoarcus* (Zhou *et al.*, 1995) of the beta-*Proteobacteria* subclass. Some sulfate-reducing bacteria (SRB) are believed capable of degrading phenol (Boopathy 1997). However, information related to the microbial characteristics of methanogenic sludge degrading phenol in wastewater is still very limited.

In this study, microbial properties of a methanogenic granular phenol-degrading sludge was characterized using the 16S rRNA/DNA-based techniques, including polymerase chain reaction (PCR) amplification, denaturing gradient gel electrophoresis (DGGE), cloning, DNA sequencing, and fluorescence *in situ* hybridization (FISH). The sludge was sampled from an upflow anaerobic sludge blanket reactor, which removed 98% of phenol (up to 1260 mg/l) in wastewater at 26°C with 12 hours of hydraulic retention (Fang *et al.*, 2004).

Materials and methods

Sludge sample

A methanogenic phenol-degrading sludge was sampled from a 2.8 litre UASB reactor. Treating a wastewater containing 1260 mg/l of phenol, corresponding to a COD loading rate of 6.0 g-COD/(l·d), at pH 7.0–7.5 and 26 ± 1°C, the reactor consistently removed 98% of phenol from wastewater under steady-state condition. Details of the reactor conditions and performance have been reported previously (Fang *et al.*, 2004).

DNA extraction, PCR, DGGE, cloning, sequencing and phylogenetic analysis

A 5 ml sludge sample was homogenized and the DNA was extracted using the method described previously (Zhang and Fang, 2001). Fragments of 16S rDNA were amplified by PCR (Zhang and Fang, 2001) using the *Eubacteria* specific primer set of EUB8F (5′-AGAGTTTGATCMTGGCTCAG) and UNIV1492R (5′-GGTTACCTTGTTACGACTT) in an automated thermal cycler (GeneAmp® PCR 9700, Perkin-Elmer, Foster City, CA) following a three-step program: (1) initial denaturation (94 °C, 7 min), (2) 35 cycles of denaturation (92 °C, 1 min), annealing (54 °C, 1 min) and extension (72 °C, 1 min), and (3) final extension (72 °C, 10 min). The amplified products were then cloned using the TA Cloning Kit (Invitrogen, Carlsbad, CA) (Zhang and Fang, 2001). A total of 90 clones were selected for the plasmids recovery. The inserted fragment in the plasmids was sequenced using an auto sequencer (ABI 377A, Perkin-Elmer, Foster City, CA) and dRhodamine Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin-Elmer, Foster City, CA) with the primer set of M13 Rev (5′-GTTTGATCCTGGCTCAG), M13 Univ (5′-CAGGAAACAGCTATGAC) and 1055R (5′-CACGAGCTGACGACAGCCAT). Finally, the sequences with over 99% similarity were grouped into operational taxonomy units (OTU).

The obtained DNA sequences and the closest 16S rDNA sequences of reference microorganisms retrieved from the GenBank were aligned and checked manually using the BioEdit (Hall, 1999). Phylogenetic trees were then constructed using the neighbor-joining method with MEGA 2.1 (Kumar *et al.*, 1993).

Fluorescence *in situ* hybridization

The sludge sample was fixed as described previously (Zhang *et al.*, 2003). The fixed sludge was subsequently subjected to five freeze-thaw cycles (−80 °C and +40 °C) to improve the penetration efficiency of the oligonucleotide probes. The fixed sample was embedded with Tissue Tek OCT compound (Torrance, CA, USA), and sectioned into 20 μm thickness with a Cryostat 1510 (Leica, Germany) at −20°C. The sectioned sludge samples were collected onto a glass slide coated with 0.01% poly-L-lysine solution, dehydrated sequentially using 50%, 80% and 96% ethanol solutions.

After drying at room temperature, the fixed samples immobilized on the microscopic slides were hybridized (Amann *et al.*, 1995) in a mixture containing 1 μl each of individual probe solutions (100 ng/μl) and 10 μl of pH 7.2 hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl) for 90 min at 46 °C in a sealed moisture chamber. The buffer also contained formamide at various concentrations depending on the probe used: ARC915 20%, MG1200 10%, MB1174 35%, MX825 20% and EUB338 0–80%. Samples on slide were then incubated in the pH 7.2 washing buffer (0.07 M NaCl, 0.01% sodium dodecyl sulfate, 20 mM Tris-HCl, 5 mM EDTA) for 30 min at 48 °C. The slides were then washed briefly with distilled water and air dried before examination using a Zeiss LSM 5 Pascal confocal laser scanning microscope (Zeiss, Jena, Germany). This system was equipped with two laser sources, Ar and He-Ne, and two objectives, Fluar 10X NA0.5 and Plan-Apochromat 63X NA1.4. The FISH images were

captured using the multi-track mode, in which sample was sequentially excited with laser at 488 nm and 543 nm. Five microscopic fields were examined for each hybridized sample, and the population abundance in each field was analyzed using MetaView™ (Universal Imaging Corporation, USA), based on the color areas corresponding to the specific probes.

Accession numbers

The nucleotide sequence data reported in this paper have been assigned by the GenBank the following accession numbers: AY261808-820.

Results and discussion

The abundances of *Eubacteria* and methanogens were estimated from a series of FISH images. Figures 1a and 1b illustrate the non-layered distribution of both microbes in the cross-section of a typical phenol-degrading granule, whereas Figs. 1c and 1d are close-up images of individual cells. Overall, the sludge was composed of 26 ± 6% *Eubacteria* and 74 ± 9% methanogens, of which 54 ± 6% were acetotrophic *Methanosaetaceae*, 14 ± 3% and 3 ± 2% were hydrogenotrophic *Methanomicrobiales* and *Methanobacteriaceae*, respectively.

Based on the 16S rDNA analysis of 90 clones, the *Eubacteria* in the sludge was composed of 13 OTUs. Figure 2 illustrates the phylogenetic tree of the OTUs in the phenol-degrading sludge and their close relatives based on full 16S rDNA sequences.

Five OTUs, i.e. PD-UASB-5 (33% of total clones), −35 (4%), −40 (6%), −54 (3%) and −57 (2%), were affiliated with the *Syntrophus* genus and its related environmental clones. These include four clones obtained from a methanogenic benzoate-degrading consortium, i.e. BA003 (AF323758), BA039 (AF323763), BA053 (AF323776) and BA044 (AF323776), and an unidentified clone vadinBC38 from an anaerobic digester (Godon *et al.*, 1997). Many *Syntrophus* species, including *S. gentianae* (Caroline *et al.*, 1998), *S. buswellii* (Caroline *et al.*, 1998) and *S. aciditrophicus* (Bradley *et al.*, 1999; Elshahed and McInerney, 2001), are known benzoate degraders. *S. buswellii* was also found responsible for the benzoate degradation in a methanogenic sludge (Fang *et al.*, 1996).

PD-UASB-10 (24% of the total clones) is related (similarity 94–97%) to several unidentified or uncultured bacteria, including an anaerobic bacterium 'strain 7' (Letowski *et al.*, 2001), clone SHA-86 obtained from an anaerobic community dechlorinated 1,2-dichlorpropane (AJ306756), plus two uncultured bacteria WCHB1-20 and WCHB1-89 found in a contaminated aquifer (Dojka *et al.*, 1998). PD-UASB-10, anaerobic bacterium 'strain 7', SHA-86, WCHB1-20 and WCHB1-89 appears to form a distinct group in the

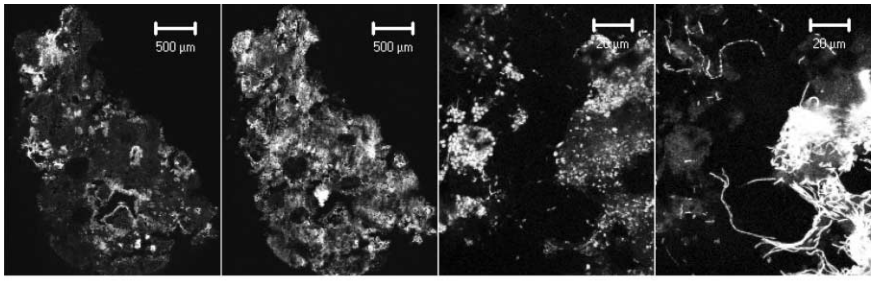

Figure 1 FISH images illustrating the non-layered distribution of: (a) *Eubacteria*, and (b) methanogens in a cross-section of a typical phenol-degrading granule, and the individual cells of (c) *Eubacteria*, and (d) methanogens in close-up images

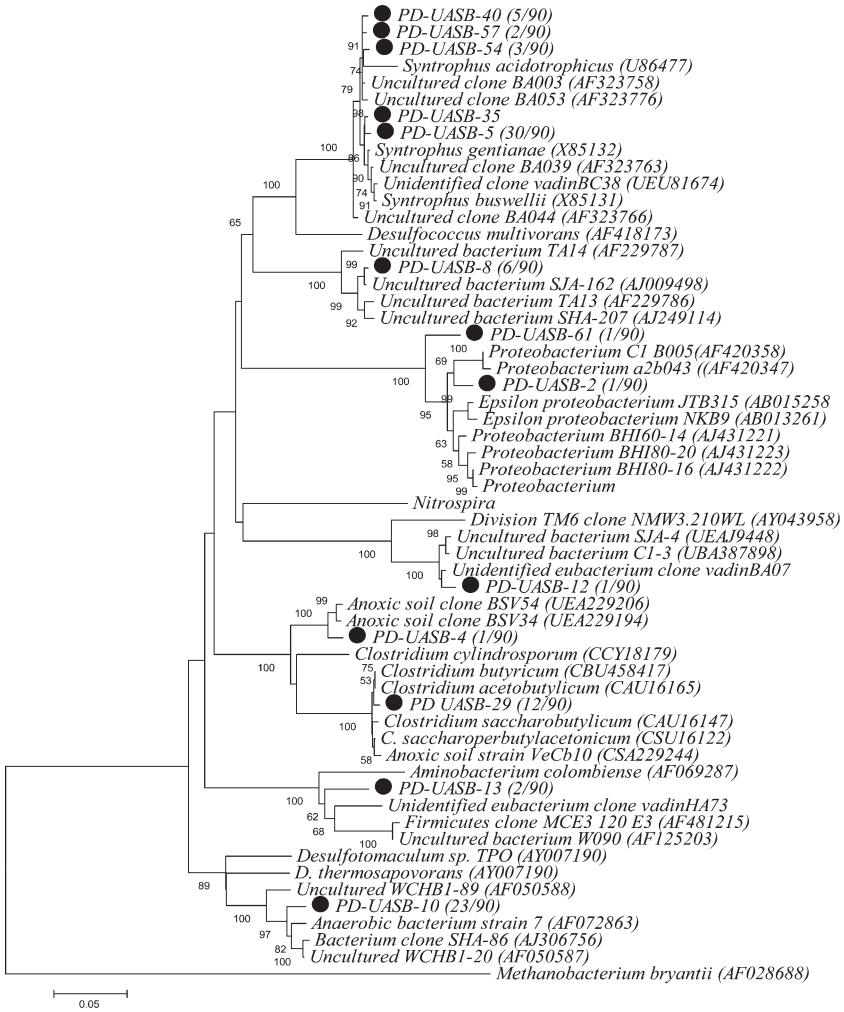

Figure 2 Phylogenetic tree of the OTUs in the phenol-degrading sludge and their close relatives based on full 16S rDNA sequences. The tree was based on Jukes–Cantor distance and constructed using the neighbor-joining algorithm with 1000 bootstrapping. *Methanobacterium bryantii* was selected as the outgroup species. The scale bar represents 0.05 substitution per nucleotide position. Numbers at the nodes are the bootstrap values. The accession number of each species was shown in parenthesis

Firmicutes division. It is remotely related to SRB, the closest of which being *Desulfotomaculum thermosapovorans* with only 90% similarity. Some species of *Desulfotomaculum* are capable of degrading benzoate (Plugge *et al.*, 2002).

PD-UASB-29 (13% of total clones) is affiliated with the genus *Clostridium*, the closest species including *C. saccharoperbutylacetonicum* (97%), *C. saccharobutylicum* (96%), *C. acetobutylicum* (96%), *C. butyricum* (96%), and an uncultured *Clostridium* strain VeCb10 from an anoxic bulk soil (96%). Some *Clostridium* species are capable of degrading phenol and chlorophenol (Tartakovsky *et al.*, 2001).

The OTU PD-UASB-8 (7% of total clones), had high similarity to several uncultured environmental clones, including SJA-162 (99% similarity) found in a trichlorobenzene-transforming community (von Wintzingerode *et al.*, 2000), SHA-207 (98%) in a 1,2-dichloropropane-dechlorinating consortium (Schlotelburg *et al.*, 2000) and TA13 (98%) in a terephthalate-degrading anaerobic granular sludge (Wu *et al.*, 2001). It had very low similarity to SRB, the closest of which being *Desulfococcus multivorans* with only 87%

similarity. It seems that this OTU formed a separate group with its affiliated environmental clones in the subdivision of delta-*Proteobacteria*. The five remaining OTUs, i.e. PD-UASB-13 (2% of total clones), −4 (1%), −2 (1%), −12 (1%) and −61 (1%) have 91–98% similarity with several environmental clones. The role of these six OTUs in phenol degradation remains unclear.

Fang *et al.* (1996) showed that phenol could be anaerobically degraded to methane via benzoate. In this study, the degradation of phenol to benzoate was not carried out by the most abundant OTU, PD-UASB-5, judging from the physiological properties of its closest related species *Syntrophus gentianae*. Instead, phenol was more likely degraded to benzoate by the second and third most abundant OTUs, i.e. PD-UASB-10 and −29. Letowski *et al.* (2001) showed that the degradation of phenol to benzoate was not carried out by individual strains, but by the co-culture of the anaerobic bacterium 'strain 7' and a *Clostridium*-like 'strain 6'. In this study, PD-UASB-10 has 98% similarity to several *Clostridium* species, and PD-UASB-29 is related to the anaerobic bacterium 'strain 7' with 97% similarity. Thus, phenol was likely converted by PD-UASB-10 and −29 jointly into benzoate, which was then further degraded by *Syntrophus* species, such as PD-UASB-5, to acetate and H_2/CO_2. Methanogens lastly converted acetate and H_2/CO_2 into methane.

Conclusions

Based on DNA analysis, the methanogenic granular phenol-degrading sludge was composed of 26 ± 6% *Eubacteria* and 74 ± 9% methanogens. The *Eubacteria* was composed of 13 OTUs. Of which one OTU resembling *Clostridium* and the other remotely resembling *Desulfotomaculum*, were likely responsible for the conversion of phenol to benzoate, which was further degraded by five OTUs resembling *Syntrophus* to acetate and H_2/CO_2; methanogens lastly converted acetate and H_2/CO_2 into methane. The role of six remaining OTUs remains unclear. The methanogens included 54 ± 6% acetotrophic *Methanosaetaceae*, 14 ± 3% and 3 ± 2% hydrogenotrophic *Methanomicrobiales* and *Methanobacteriaceae*, respectively.

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