Study of microbial community of brewery-treating granular sludge by denaturing gradient gel electrophoresis of 16S rRNA gene

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Abstract The microbial community structure of granular sludge from an upflow anaerobic sludge blanket (UASB) reactor treating brewery effluent was studied by denaturing gradient gel electrophoresis (DGGE). Twelve major bands were observed in the DGGE fingerprint for the Bacteria domain and four bands for the Archaea domain. Of the bacterial bands observed, six were successfully purified and sequenced. Among them, three were related to the gram-positive low G +C group, one to the Delta subclass of the Proteobacteria, one to the Gamma subclass, and one to the Cytophaga group with no close related sequence. The 16S rRNA sequences of the four archaeal bands were closely associated with Methanosaeta concilii and Methanobacterium formicum.

Keywords USAB; DGGE; granule; microbial community; 16S rRNA

Introduction

The upflow anaerobic sludge blanket (USB) technology has been widely used for industrial wastewater treatment in the last two decades (Lettinga et al., 1980; Fang and Chui, 1993). Bacteria in USB reactors aggregate to form granules with diameters up to 3–4 mm. Inside each granule, which can be regarded as a micro-ecosystem, fermentative bacteria degrade complex organic substances to smaller molecules. These intermediates are then further degraded by acidogens to volatile fatty acids, which are in turn converted into methane by methanogens. Many microbes participate in such a complex process. Some of the bacterial species which are critical in syntrophic degradation, such as Syntrophobacter wolinii (Boone and Bryant, 1980), Syntrophus buswellii (Mountfort et al., 1984) and Syntrophus gentianae (Schocke and Schink, 1977), have been isolated. However, the traditional cultivation could lead to bias in overlooking of the presence of many microbes (Head et al., 1998).

Advanced molecular techniques have been shown to be a promising tool to study the microbial community structure in various natural environments (Amann et al., 1995; Pace, 1996; Head et al., 1998). More recently, these techniques have also been applied to the study of microbial population in some USAB granules (Sekiguchi et al., 1998; Rocheleau et al., 1999). In this study, molecular techniques were used to investigate the microbial community of UASB granules treating brewery wastewater. This type of granules exhibited a three-layered microstructure (Fang et al., 1995a, 1995b). The outer layer was populated with bacteria with various morphologies, including single and tetrad cocci, bacilli and long-rod cells. The middle layer was composed of juxtapositioned syntrophic microcolonies and the centre core was densely packed with Methanosaeta-like bacteria. The denaturing gradient gel electrophoresis (DGGE) method, which utilizes the melting properties of each unique DNA fragment in a denaturing gradient gel to separate different double-stranded DNA fragments with an identical length (Muyzer et al., 1993; Ferris et
al., 1996, 1997), was used to obtain the 16S rRNA genetic fingerprint. Major DGGE bands of the Bacteria and Archaea domains detected were purified and sequenced, and their phylogenetic affiliations were determined.

Materials and methods

Reactivation of anaerobic granular sludge

The granular sludge treating brewery effluent was preserved from a previous study (Fang et al., 1995a). The sludge was kept in a 4°C refrigerator for four years prior to this study. In order to ensure a healthy microbial population, the granules were re-activated by feeding with brewery effluent. The organic loading rate was slowly increased from 0.1 g-COD/(d.g-VSS) to 2.0 g-COD/(d.g-VSS) in 8 weeks. The bioactivity of the granular sludge was monitored by the production of methane (Fang and Chui, 1993). After one week of activation, the specific methane production rate was 8 ml/(d.g-VSS). It was increased to 74 ml/(d.g-VSS) after three weeks, and finally stabilized at the maximum level of 370 ml/(d.g-VSS) after 5 weeks. The granular sludges were sampled on days 1, 7, 21 and 56 for analysis.

DNA extraction, PCR amplification and DGGE

The sludge samples were mechanically homogenized using a mini-beadbeater (BioSpec), followed by DNA extraction (Stahl et al., 1988). 16S-rRNA genes from the purified DNA were amplified using polymerase chain reaction (PCR) with two sets of PCR primers for DGGE analysis. The primer set specific to the Bacteria domain was EUB96 (5'-AACGC-GAAGAACCTTAC-3') with a GC-clamp (5'-CGCCGGGGGCGCGCCCCGGGCCGGGGGCGGGGCACGCGGGG-3') and UNIV1392R (5'-ACGGGCGGTGTGTGC-3') (Stahl et al., 1988). The other primer set specific to the Archaea domain was ARC622FGC (5'-TGAAATCYYRTAATCCC-3') with a GC-CLAMP (5'-CGCCGGGGGCGCGCCCCGGGCCGGGGGCGGGGCACGCGGGG-3'), and ARC915R (5'-GTGCTCCCCGCCAATTCCT-3'). The PCR program was performed in a 2400 thermal cycler (Perkin-Elmer, Norwalk, Conn.) using the following program: initial denaturation at 94°C for 10 min; 30 cycles of denaturation (94°C, 1 min), annealing (54°C and 56°C, respectively, for Bacteria and Archaea specific primers, 1 min), and extension (72°C, 1 min); and final extension at 72°C for 10 min. PCR products were examined on ethidium bromide-stained agarose gels, and were used for DGGE analysis.

DGGE of the PCR-amplified 16S rDNA was performed with the D-Code system (BioRad Laboratories, Hercules, Calif.) (Muyzer et al., 1993). The polyacrylamide concentrations used in the analysis of DGGE were 6% and 8% for bacterial and archaeal primers, respectively. And, the denaturing gradients were 40–60% and 45–65%. The denaturing gradient gel was then electrophoresed at 200 V at 60°C for 5 hours. Afterwards, gels were stained with ethidium bromide (0.5 mg/l) and photographed under UV transillumination.

Phylogenetic analysis of DGGE bands

Dominant DGGE bands were cut, and the DNA was eluted and recovered according to the standard procedure (Maniatic et al., 1989). The recovered DNA was used as the DNA template in the PCR reaction with the aforementioned primer sets. Following the amplification, the product was re-run on DGGE gel. The purification procedure of the DGGE bands was repeated to ensure that a single DNA fragment was obtained. The PCR Product was then sequenced by a DNA sequence analyzer (PRISM, model 377 Applied Biosystem). The partial 16S rRNA sequences obtained were compared with those in the GenBank by use of the NCBI Blast program. Closely related sequences were retrieved and aligned with those DGGE band sequences using the CLUSTAL W package. Phylogeny trees were constructed by the neighbor-joining method provided in the MEG package (Saito and Neil, 1987).
Community structure fingerprints of the bacterial population in the four samples taken during re-activation were analyzed using DGGE. Figure 1a illustrates that the number, position and relative intensity of the DGGE pattern of the Bacterial domain observed in all samples taken from day 1 to day 56 were very similar. Likewise, the pattern of the Archaeal domain, as illustrated in Figure 1b, for the two samples taken on days 1 and 5 were nearly identical. These results indicated that the microbial community in both Bacteria and Archaea domains did not change during the re-activation, and most likely the microbial community had remained intact over four years of storage at 4°C. The DGGE fingerprint in Figure 1a further indicates that at least 12 bacterial populations were present in the USAB granules. On the other hand, Figure 1b shows that the archaeal fingerprint was less complex and contained only four archaeal populations.

In order to further understand the composition of the microbial community in the UASB granules, major bacterial and archaeal DGGE bands were purified, and the DNA sequence in each band was determined. Of the 12 bacterial bands, six were successfully purified and sequenced. The phylogenetic analysis of these six 16S rRNA sequences is shown in Figure 2. The partial sequences (~400 bp) of DGGE bands eub4 and eub5, which differ only in one nucleotide, were closely associated with Clostridium sporosphaeroides and Ruminococcus bromii (~94% similarity) from the family Clostridiaceae. This result indicates that either closely related bacteria were present in the UASB granule, or a variation occurred among those copies of the 16S rRNA on a bacterial chromosome. Both Clostridium and Ruminococcus are known to be associated with the fermentation of carbohydrates, which are the major constituent of brewery effluent (Holt et al., 1994). Previous microscopic studies (Guiot et al., 1992; Fang et al., 1995a, 1995b) observed that the brewery-treating UASB granules had a dense skin layer consisting of a variety of bacteria. The Clostridium sp. found in this study is likely to be one of the major bacterial populations in the skin layer of
the granules. This could be further verified with fluorescent in-situ hybridisation with oligonucleotide probes targeting *Clostridium* sp.

Sequences of DGGE bands eub2 and eub3 had a lesser degree of similarity to the known sequences in GenBank. The closest matches (~89%) of band eub2 were two unidentified bacteria (RFLP5 and BB48) found in an anaerobic digester (Godon et al., 1999) and a dechlorinating microbial community (Holoman et al., 1998). The closest known species of band eub2 was *Synergistes jonesii*. Sequence of band eub3 was found to be affiliated with *Cytophaga* (88% similarity). 16S rRNA clones related to *Synergistes* and *Cytophaga* had also been reported in sludge obtained from an anaerobic digester (Godon et al., 1997) and a UASB reactor (Sekiguchi et al., 1998).

The sequence of band eub1 (402 bp) was closely related to the genus *Desulfovibrio*, and an unidentified bacterial clone vadinHA40 (similarity, 99%) (Godon et al., 1997). The clone, vadinHA40, was obtained from a fluidized-bed anaerobic digester treating winery wastewater which was also rich in carbohydrates and alcohols, similar to the brewery effluent. The presence of sulfate-reducing bacteria (SRB) sequence also supported a previous microscopic observation (Fang et al., 1995a) that a curved *Desulfovibrio*-like species was present in juxtaposition with a *Methanospirillum*-like species in UASB granules treating brewery effluent. It is known that *Desulfovibrio* is capable of degrading alcohols into propionate and H₂ when growing with methanogen in the absence of sulfate (Qatibi et al., 1991). Electron microscopic observations also suggested that closely physical association (~0.05 to 0.2 µm) of SRB and methanogen was critical to ensure an efficient interspecies transfer of hydrogen and formate (Stams, 1994). Since fermentation of alcohols proceeds only when the H₂ partial pressure is kept low (Bryant et al., 1977), syntrophic association between the hydrogen-producing *Desulfovibrio* and hydrogen-consuming methanogens is likely to occur.

\[ \text{Figure 2} \quad \text{Phylogenic tree of partial 16S rRNA gene sequences from DGGE bands for Bacteria} \]
DGGE band eub6 was found to have high sequence similarity (≥99%) to several bacterial 16S rRNA sequences, including *Lysobacter enzymogenes*, *Stentrophomonas maltophilia*, and *Xanthomonas* sp. in the gamma subdivision of the class *Proteobacteria*. This is not surprising since the partial sequence retrieved (~419 bp) was not long enough to resolve the phylogenetic affiliation among closely related bacteria sequences. The predominant bacterial partial 16S rRNA sequences found in this study were more closely matched to the sludge treating winery effluent (Godon et al., 1997) than to the sludge treating sucrose, propionate and acetate (Sekiguchi et al., 1998). This is most likely due to similarity in the composition between the brewery and the winery effluents.

The dominant Archaea DGGE bands were closely affiliated (99% similarity) with *Methanobacterium formicicum* (band arch1) and *Methanosaeta concilii* (bands arch2, arch3 and arch4). Microorganisms with similar morphologies to *M. formicicum* (rod-shaped filament) and *M. concilii* (bamboo-shaped long filament with a diameter of 0.7–0.8 µm) were both observed previously in the brewery-treating USAB granules by use of electron microscopy (Fang et al., 1995a). Especially, the predominance of *M. concilii* in UASB reactors was often reported (Sekiguchi et al., 1998; Rocheleau et al., 1999; Fang et al., 1994). Although the bacterial population found in the UASB granules of this study was highly similar to that found in the fluidized sludge by Godon et al. (1997), the archaeal population was significantly different. In the fluidized sludge, *Methanosarcina*, *Methanobacterium* and some unknown archaeal clones were present, but not *Methanosaeta*. It seems that the microstructure of the UASB biogranules might have an effect on the Archaeal population, and the *Methanosaeta* could be of importance in granule formation.

**Conclusion**

The microbial community of the UASB granules was reflected from the DGGE fingerprint of the 16S rRNA gene. After four years of storage at 4°C, the microbial community structure in general remained intact. There were twelve dominant DGGE bands of the *Bacteria* domain and four of the *Archaea* domain. Three dominant *Bacterial* bands were related to the gram-positive low G+C group, one to the *Delta* subclass of the *Proteobacteria*, one to the *Gamma* subclass, and one to the *Cytophaga* group with no close related sequence. The 16S rRNA sequences of the dominant *Archaeal* bands were closely associated with *Methanosaeta concilii* and *Methanobacterium formicicum*. High sequence similarities between DGGE bands (>99%) were found in both *Bacteria* and *Archaea* domains. The observation of a number of unidentified *Bacteria* suggested that our knowledge of the microbial community of the anaerobic sludge is still very limited.

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**References**


