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ORIGINAL PAPER

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Microbial diversity of a mesophilic hydrogen-producing sludge

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Abstract A hydrogen-producing sludge degraded 99% of glucose at 36 °C and pH 5.5, producing a methane-free biogas (comprising 64% hydrogen) and an effluent comprising mostly butyrate, acetate, and ethanol. The yield was 0.26 l H_2 g^{-1} glucose and the production rate per gram of volatile suspended solids was 4.6 l H_2 day^{-1}. A 16S rDNA library was constructed from the sludge for microbial species determination. A total of 96 clones were selected for plasmids recovery, screened by denaturing gradient gel electrophoresis, and sequenced for rDNA. Based on the phylogenetic analysis of the rDNA sequences, 64.6% of all the clones were affiliated with three *Clostridium* species (Clostridiaceae), 18.8% with Enterobacteriaceae, and 3.1% with *Streptococcus bovis* (Streptococcaceae). The remaining 13.5% belonged to eight operational taxonomic units, the affiliations of which were not identified.

Introduction

Hydrogen is an important industrial commodity (Kirk et al. 1985). It is the raw material for the synthesis of ammonia, alcohols, and aldehydes, and for the hydrogenation of various petroleum and edible oils, coal, and shale oil. In addition, hydrogen is a clean energy, which has a wide range of potential applications for the near future. Although hydrogen is traditionally produced by hydrocarbon reformation or electrolysis of water (Hart 1997), it may also be produced by micro-organisms. Studies on microbial hydrogen production has been conducted mostly using pure cultures, either natural (Kalia et al. 1994; Karube et al. 1982; Kim et al. 1984) or genetically modified (Asada et al. 2000). Conversely, hydrogen is a key intermediate in the anaerobic degradation of organic compounds and may be recovered from wastewater (Ueno et al. 1996) or solid waste (Lay et al. 1999; Mizuno et al. 1997), using mixed cultures by suppressing the activity of hydrogenotrophic methanogens. However, little information is available on the hydrogen-producing microbial community.

Traditionally, microbes are identified by isolating individual cultures and examining their physiological, biochemical, and morphological characteristics. *Clostridium* was found using these methods as the hydrogen-producing bacterium (HPB) in a mixed culture (Lay 2000). However, such an identification is often unreliable. First, microbes may not be properly isolated from the artificial growth medium. Second, many microbes grow syntrophically with others and thus cannot be cultured individually (Pike and Curds 1971; Wagner et al. 1993). Third, many microbes share similar physiological, biochemical and morphological characteristics and thus cannot be distinguished by these.

More recently, a number of molecular techniques have been developed for the qualitative and quantitative analysis of microbial communities. Among them, 16S rDNA-based methods have been extensively applied in the study of activated sludge (Nielsen et al. 1999), biofilms (Zhang and Fang 2000), ocean mats (Muyzer et al. 1993), sediments (Devereux and Mundform 1994), etc.

In this study, a mesophilic microbial community converting carbohydrate in wastewater into hydrogen was developed. Glucose was chosen as the model carbohydrate. The phylogenetic diversity of this hydrogen-producing community was analyzed using 16S rDNA-based techniques.

Materials and methods

Reactor conditions

The HPB was incubated in a complete-mix fermentor with a working volume of 1.7 l (B. Braun Biotech International, Melsunge, Germany), seeded with sludge obtained from the secondary sedimentation tank of a local wastewater treatment plant. Each liter of

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Table 1 DNA sequences of primers and GC-clamp

Primer	Sequence	Specificity	Target position	Reference
GM3F	5′-AGA GTT TGA TCM TGG CTC AG	Bacteria	8–27	Lane (1991)
GM4R	5′-GGT TAC CTT GTT ACG ACT T	Prokaryotes	1,491–1,509	Lane (1991)
EUB968F	5′-AAC GCG AAG AAC CTT AC	Bacteria	968–984	Nielsen et al. (1999)
UNIV1392R	5′-ACG GGC GGT GTG TRC	Universal	1,392–1,406	Nielsen et al. (1999)
ARC622F	5′-TGA AAT CYY RTA ATC CC	Archaea	622–638	Fang et al. (2001)
ARC934R	5′-GTG CTC CCC CGC CAA TTC CT	Archaea	915–934	Giovannoni et al. (1990)
M13F	5′-GTA AAA CGA CGG CCA G	pCR2.1 Vector	–	Invitrogen (1999)
M13R	5′-CAG GAA ACA GCT ATG AC	pCR2.1 Vector	–	Invitrogen (1999)
GC-clamp	5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G	–	–	Nielsen et al. (1999)

the feed medium consisted of the following: 7 g glucose, 1 g $NaHCO_3$, 500 mg NH_4Cl, 250 mg KH_2PO_4, 250 mg K_2HPO_4, 320 mg $MgSO_4 \cdot 7H_2O$, 50 mg $FeCl_3$, 32 mg $NiSO_4$, 50 mg $CaCl_2$, 7.2 mg $Na_2BO_7 \cdot H_2O$, 14.4 mg $(NH_4)_6Mo_7O_{24} \cdot H_2O$, 23 mg $ZnCl_2$, 21 mg $CoCl_2 \cdot H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$ and 30 mg $MnCl_2 \cdot 4H_2O$. The medium was kept at 4 °C, purged with nitrogen to ensure anaerobic conditions, and fed continuously by a peristaltic pump. The fermentor was kept at 36 °C in darkness to suppress photosynthetic activity. The mixed liquor was kept at pH 5.5 to suppress methanogenic activity and the hydraulic retention time was kept at 6.6 h.

The amount of biogas produced was recorded daily, using the water displacement method. The hydrogen, carbon dioxide, nitrogen, and methane contents of the biogas were analyzed by a gas chromatograph (GC; Hewlett Packard 5890 II) equipped with a thermal conductivity detector and a 2 m × 2 mm (inside diameter) stainless steel column packed with Porapak N (80–100 mesh). The injector, detector, and column temperatures were kept at 57 °C, 180 °C, and 50 °C, respectively. The concentrations of volatile fatty acids and alcohols were determined by a second GC of same model, which was equipped with a flame ionization detector and a 10 m × 0.53 mm Hewlett Packard FFAP fused-silica capillary column, following the procedures described by Yu and Fang (2000). The concentration of glucose was measured by the phenol–sulfuric acid method (Herbert et al. 1971). The volatile suspended solids (VSS) were measured according to standard methods (APHA 1992).

DNA extraction

A 10-ml sludge sample was repeatedly frozen (–80 °C) and thawed (37 °C) four times. The sample was then centrifuged at 4,000 rpm for 5 min to collect the bacterial cells. Pellets were resuspended in 1 ml of a pH 8.0 lysis buffer (0.75 M sucrose, 100 mM EDTA, 100 mM Tris) and homogenized in a Mini-Beadbeater (Biospec Products, Bartlesville, Okla.) with 0.2 g of 0.1 mm glass bead at 5,000 rpm for 10 s. To the homogenized sample was added 4 ml of lysis buffer, 100 µl each of lysozyme solution (50 mg ml^{-1}) and achromopeptidase solution (10 mg ml^{-1}) and the mixture was incubated at 37 °C for 30 min, followed by the addition of 0.5 ml of 10% lauryl sulfate and 50 µl of proteinase K solution (20 mg l^{-1}). The mixture was further incubated at 37 °C for 2 h, before adding 0.8 ml of 5 M NaCl solution and 0.6 ml of a mixed solution containing 10% cetyltrimethylammonium bromide and 4.1% NaCl, followed by another 20 min of incubation at 65 °C. The solution was then mixed with a 6.6 ml solution of phenol/$CHCl_3$/isoamyl alcohol (25:24:1) and centrifuged at 4,000 rpm for 20 min. The supernatant (5.0 ml) was mixed with an equal volume of $CHC1_3$/isoamyl alcohol (24:1) solution and centrifuged again. The supernatant (4.0 ml) was mixed with 2.4 ml isopropanol and kept at 4 °C overnight. The DNA precipitates were centrifuged the next day at 12,000 rpm for 10 min and then rinsed with 0.8 ml of 70% ethanol. After another centrifugation, the DNA precipitates were air-dried for 5 min, before being dissolved in 100 µl of water.

PCR amplification

Table 1 lists the DNA sequence, specificity, and target position in *Escherichia coli* 16S rDNA of eight primers (Gibco BRL, Gaithesburg, Md.) used in this study. Among them, the primer sets GM3F/GM4R and EUB968F/UNIV1392R were applied in PCR for bacteria while the primer set ARCH622F/ARCH934R was used in PCR for archaea. All PCR amplifications were conducted in 30 µl of a pH 8.3 buffer (Pharmacia Biotech, Piscataway, N.J.) containing 200 µM each of the four deoxynucleotide triphosphates, 15 mM $MgCl_2$, 0.1 µM of individual primers, and 1 unit of *Taq* polymerase (Pharmacia Biotech, Piscataway, N.J.). An automated thermal cycler (GeneAmp PCR 9700; Perkin-Elmer, Foster City, Calif.) was used for PCR amplification, using the following program for denaturing gradient gel electrophoresis (DGGE): an initial denaturation at 94 °C for 7 min, 35 cycles of denaturation (1 min at 92 °C), annealing (1 min at 54 °C or 56 °C for bacteria or archaea, respectively) and extension (1 min at 72 °C), and a final extension at 72 °C for 10 min before storage at 4 °C. The PCR-amplified DNA fragments for cloning followed the same procedures, except they received only 25 cycles of denaturation, annealing, and extension.

DGGE screening

The presence of archaea and bacteria was checked individually before DGGE screening. A 10-µl PCR-amplified product solution was loaded onto a 1% agarose gel in a pH 7.4 TAE buffer (20 mM Tris-acetate, 10 mM sodium acetate, 0.5 mM disodium EDTA), followed by staining with ethidium bromide. The staining result was negative for archaea, indicating its absence from the mixed culture. Consequently, the archaea population was not further analyzed in this study.

In contrast, the staining result was positive for bacteria. Thus, DGGE screening was conducted for the PCR-amplified bacterial DNA fragments of 478 base pairs, using the primer set of EUB968F plus GC-clamp and UNIV1392R. The DGGE was conducted following the method of Muyzer et al. (1993) using a DCode universal mutation detection system (BioRad, Hemel Hempstead, UK). After silver-staining, each band in the DGGE profile represented a DNA fragment from an individual bacterium. The DGGE results showed more than eight visible bands in the profile.

Cloning

Longer bacterial DNA fragments of 1,501 base pairs were PCR-amplified using the primer set of GM3F and GM4R. The PCR-amplified products were cloned using the TA cloning kit (Invitrogen, Carlsbad, Calif.) following the manufacturer's instructions. Due to the complexity of the DGGE profile of bacterial DNA fragments, a total of 96 colonies were selected for inoculation in a 1.0-ml Luria–Bertani medium containing 50 mg kanamycin l^{-1}.

Table 2 Phylogenic affiliation of operational taxonomic units

OTU	Sequence length	Phylogenetic relationship			No. of clones	Abundance (%)
		Family	Closest species in GenBank	Similarity (%)		
HPB-G1-11	471	Clostridiaceae	*Clostridium cellulosi*	95	24	25.0
HPB-G1-15	748	Clostridiaceae	*C. cellulosi*	94	12	12.5
HPB-G1-5	593	Clostridiaceae	*C. cellulosi*	90	6	6.3
HPB-G1-6	657	Clostridiaceae	*C. acetobutylicum*	96	12	12.5
HPB-G1-8	511	Clostridiaceae	*C. tyrobutyricum*	95	8	8.3
HPB-G1-14	346	Streptococcaceae	*Streptococcus bovis*	96	3	3.1
HPB-G1-4	531	Enterobacteriaceae	*Citrobacter* sp.	92	18	18.8
Eight more OTUs with one or two clones each				–	13	13.5
Total					96	100.0

After 18 h of incubation of the inoculated clones at 37 °C, the plasmids were recovered (Sambrook et al. 1989), and dissolved in 50 µl of pure water.

A plasmid of each individual clone was amplified using the primer set M13F/M13R, following the aforementioned PCR program. Each set of PCR products was further analyzed by DGGE.

DNA sequencing and phylogenic analysis

DGGE profiles of the PCR amplified fragment of the 96 clones, amplified using the primer set of EUB968F with GC clamp and UNIV1392R, were compared. A total of 15 bands were identified in the DGGE profiles. Seven of them were shared by three or more clones. The DNA of these clones were then sequenced using an auto sequencer (ABI model 377A; Perkin-Elmer, Foster City, Calif.) and dRhodamine terminator cycle sequencing FS ready reaction kit (Perkin-Elmer, Foster City, Calif.). Each DNA sequence was checked using the check-chimera program (Maidak et al. 1997) to detect possible chimeric artifacts, which (if any) were then excluded from further analysis. All sequences were compared with those of the reference micro-organisms available in the GenBank database by BLAST search (Altschul et al. 1990). The closest 16S rDNA sequences of reference micro-organisms were retrieved from the GenBank and aligned using the Clustal W software (Thompson et al. 1994) to construct phylogenetic trees, using the neighbor-joining method (Saito and Nei 1987) by MEGA 2.1 (Kumar et al. 1993). Bootstrap re-sampling analysis (Felsenstein 1985) for 500 replicates was performed to estimate the confidence of tree topologies.

Accession numbers

The DNA sequence data reported in this paper will appear in the GenBank, EMBL, and DDBJ nucleotide databases under the accession numbers AF284555 (HPB-G1-11), AF284556 (HPB-G1-15), AF284557 (HPB-G1-4), AF284558 (HPB-G1-5), AF284559 (HPB-G1-6), AF284560 (HPB-G1-8), and AF284561 (HPB-G1-14).

Results

Hydrogen production

The fermentor was operated for over 60 days at 36 °C, pH 5.5 with 6.6 h of hydraulic retention. After day 30, the fermentor reached a steady state, judging from its composition, the production rate of biogas, and the volatile fatty acids and residual glucose concentrations in the effluent. During days 30–60, the fermentor degraded 99% of the glucose, producing a methane-free biogas, comprising 64% hydrogen, 34% carbon dioxide, and 2% nitrogen, and an effluent comprising mostly butyrate (35%), acetate (29%), and ethanol (10%). Degrading 1 g of glucose produced 0.26 l of hydrogen, which is comparable to the values (0.25–0.29 l) reported for the pure culture of *C. butyricum* strain SC-E1 (Kataoka et al. 1997). The specific hydrogen production rate was 4.6 l H_2 g^{-1} VSS day^{-1}, substantially higher than the 2.2 l H_2 g^{-1} VSS day^{-1} reported for the conversion of starch at pH 5.2 at 17 h of hydraulic retention (Lay 2000).

Phylogenetic analysis

Table 2 shows that seven major operational taxonomic units (OTUs) represented a total of 83 clones (86.5% of total), and the remaining 8 OTUs represented only one or two clones each. Table 2 also lists the sequence length, number of clones and relative abundance of each of the seven major OTUs, plus the closest species found in the GenBank and the degree of similarity by BLAST analysis. Each sequence covered regions V6, V7, and V8 of 16S rDNA, except for OTU HPB-G1-14, which covered only regions V7 and V8. The OTUs had only 90–96% similarity to the closest known species. This is likely due to the fact that only a small number of hydrogen-producing bacteria have identified so far.

Results in Table 2 show that 65 16S rDNA clones (67.7% of the total clones) were affiliated with the low G+C, Gram-positive bacteria. Of these, 62 clones (64.6%) were affiliated with three *Clostridium* species in the family Clostridiaceae, and 3 clones (3.1%) were affiliated with the species *Streptococcus bovis* in the family Streptococcaceae. Another 18 clones (18.8%) were affiliated with the family Enterobacteriaceae (kingdom Proteobacteria). The remaining 13 clones (13.5%) belonged to 8 OTUs, the affiliations of which were not identified.

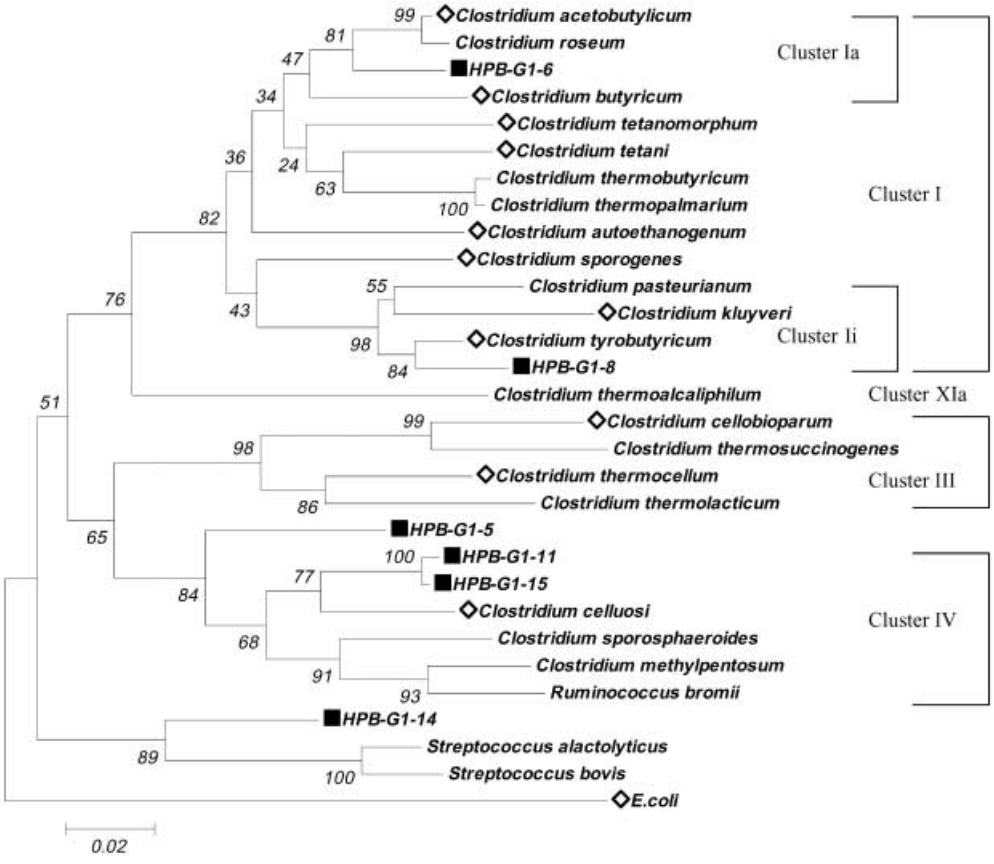

Fig. 1 Phylogenetic tree of six major operational taxonomic units (OTUs) and their close relatives in the families Clostridiaceae and Streptococcaceae, based on 340 nucleotides in the 16S rDNA sequence. Clusters Ia, Ii, III, XIa, and IV are rDNA clusters in the genus *Clostridium*. The tree is based on the Jukes–Cantor distance and was constructed using a neighbor-joining algorithm with 500 bootstrappings. *Escherichia coli* was selected as the outgroup species. The *scale bar* represents 0.02 substitutions per nucleotide position. *Numbers at the nodes* are the bootstrap values (%). ■ OTUs obtained in this study, ◇ known hydrogen-producing bacterium (HPB) species

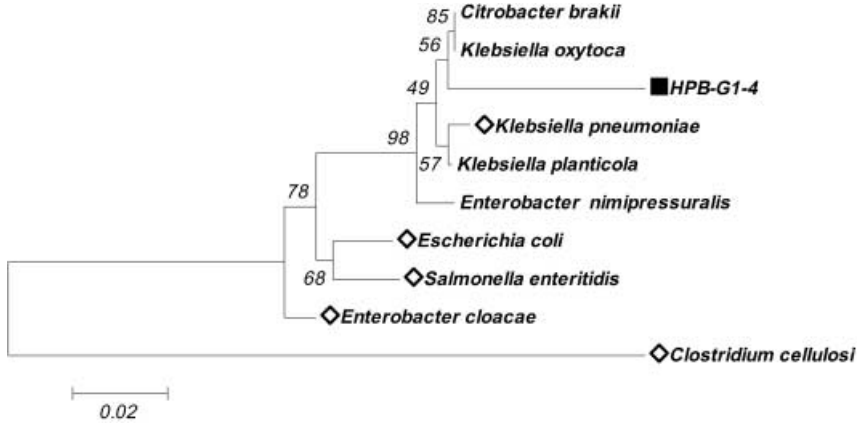

Fig. 2 Phylogenetic tree of the OTU HPB-G1-4 and its close relatives in family Enterobacteriaceae, based on 530 nucleotides in the 16S rDNA sequence. The tree is based on the Jukes–Cantor distance and was constructed using a neighbor-joining algorithm with 500 bootstrappings. *C. cellulosi* was selected as the outgroup species. The *scale bar* represents 0.02 substitutions per nucleotide position. *Numbers at the nodes* are the bootstrap values (%). ■ OTU obtained in this study, ◇ known HPB species

Phylogenetic trees were constructed for the two major OTU groups and their respective close relatives, using the neighbor-joining algorithm with 500 bootstrappings. Figure 1 illustrates the phylogenetic tree for 5 OTUs closely related to the Clostridiaceae and Streptococcaceae, based on 340 nucleotides. Figure 2 illustrates the phylogenetic tree for the OTU closely related to the Enterobacteriaceae, based on 530 nucleotides.

Discussion

Bacteria in the mesophilic HPB community

Results in Table 2 and Figure 1 show that three OTUs, HPB-G1-5, HPB-G1-11, and HPB-G1-15, representing 43.8% of the clones, are most closely related to the thermophilic bacterium *C. cellulosi* (He et al. 1991), even though the sludge was enriched under mesophilic conditions in this study. However, based on 16S rDNA data available in the GenBank, *C. cellulosi* is most closely related to another mesophilic species, *C. sporosphaeroides*, instead of thermophilic *Clostridium* spp, such as

C. thermoalcaliphilum, *C. thermobutyricum*, *C. thermocellum*, *C. thermolacticum*, *C. thermopalmarium*, and *C. thermosuccinogenes*, as shown in Figure 1. This indicates that mesophiles and thermophiles are not easily distinguished phylogenetically, as reported by Stackebrandt and Rainey (1997).

Figure 1 also shows that OTUs HPB-G1-11 and HPB-G1-15 are most closely related to each other, with a node bootstrap value of 100%. They have a 99.6% similarity, based on 471 nucleotides. These two OTUs are also distantly related with OTU HPB-G1-5. The similarity of OTUs HPB-G1-5 and HPB-G1-15 is 94.3%, based on 593 nucleotides.

OTU HPB-G1-6, representing 12 clones (12.5%), is most closely related to *C. acetobutylicum*, with 96% similarity. *C. acetobutylicum* has the optimum growth temperature of 37 °C, uses carbohydrate as carbon and energy source and produces H_2 as well as acetate, butyrate, and *n*-butanol (McCoy and Fred 1941). All of these characteristics of *C. acetobutylicum* are shared by the hydrogen-producing sludge of this study. Figure 1 shows that OTU HPB-G1-6 forms a group with *C. acetobutylicum* and *C. roseum* in the rDNA cluster Ia of *Clostridium* spp, which also comprises another HPB, *C. butyricum*.

OTU HPB-G1-8, representing eight clones (8.3%), is most closely related to another HPB, *C. tyrobutyricum* (Hippe et al. 1989; Su and Ingham 2000), which has an optimum growth temperature of 37 °C and uses monosaccharides, such as glucose and fructose, as substrate. Both OTU HPB-G1-8 and *C. tyrobutyricum* are also closely related to *C. pasteurianum* and the HPB *C. kluyveri*, with a node bootstrap value of 98%.

OTU HPB-G1-14, representing three clones, has 96% similarity with *S. bovis*. Both form a cluster with *S. alactolyticus* in the family Streptococcaceae with a node bootstrap value of 89%. Streptococcaceae are facultatively anaerobic bacteria producing mostly lactate by fermentation. They grow at 25–45 °C in pairs or in chains. Some species of Streptococcaceae were isolated from termite intestine along with *Clostridium* (Eutick et al. 1978).

Figure 2 illustrates the phylogenetic tree for OTU HPB-G1-4 and related species of Enterobacteriaceae, many of which are known as HPB, including *Klebsiella* sp., *K. pneumoniae*, *E. coli*, *Citrobacter intermedium*, *Enterobacter* sp., and *Salmonella enteritidis* (Narendra and Debabrata 2000; Solomon et al. 1995). OTU HPB-G1-4 is closely affiliated with species of *Citrobacter*, *Enterobacter*, and *Klebsiella*, with a node bootstrap value of 98%. However, it is distinctly different from *Citrobacter* and *Klebsiella*. This implies that HPB-G1-4 might be a species of a new group in the family Enterobacteriaceae. Whether OTU HPB-G1-4 produces hydrogen or not is still unclear.

Microbial diversity of HPB

Most studies on the microbial production of hydrogen used glucose as the substrate (Kataoka et al. 1997; Lin and Chang 1999; Majizat et al. 1997; Nandi and Sengupta 1998; Taguchi et al. 1993; Zhu et al. 1999) and used pure cultures, such as *Clostridium beijerinckii* (Taguchi et al. 1993), *C. butyricum* (Kataoka et al. 1997), *Escherichia coli* (Nandi and Sengupta 1998), *Rhodobacter sphaeroides* (Zhu et al. 1999) and *Enterobacter aerogenes* (Rachman et al. 1998). Little information is available on hydrogen production from glucose by a mixed culture.

Many bacteria are capable of producing hydrogen (Kalia et al. 1994; Mitsui 1975; Miyake et al. 1999; Nandi and Sengupta 1998). They are distributed across 10 of the 35 bacterial groups (Holt et al. 1994), including Gram-negative, aerobic/microaerobic rods and cocci (group 4), Gram-negative, facultatively anaerobic rods (group 5), Gram-negative, anaerobic, straight, curved, and helical bacteria (group 6), dissimilatory sulfate- or sulfur-reducing bacteria (group 7), anoxygenic, phototrophic bacteria (group 10), Gram-positive cocci (group 17), Gram-positive, endospore-forming rods and cocci (group 18), Gram-positive, regular, non-sporing rods (group 19), Gram-negative, irregular, non-sporing rods (group 20), and methanogens (group 31). However, the microbial community in this study was not diverse. A total of 64.6% of the total clones were affiliated with three species of Clostridiaceae (in group 18) and 18.8% with a species of Enterobacteriaceae (in group 5). The lack of diversity in the microbial population of this study was probably due to the tightly controlled growth conditions, with specific pH, temperature, and substrate.

HPB may be classified into four major groups: strictly anaerobic, facultative anaerobic, aerobic, and photosynthetic bacteria (Nandi and Sengupta 1998). In this study, the strictly anaerobic *Clostridium* was found to be most abundant in the sludge, followed by two facultatively anaerobic bacteria of the Enterobacteriaceae and Streptococcaceae. Aerobic and photosynthetic bacteria were absent from the mixed culture, as expected, because of the strictly anaerobic and dark conditions. Although many HPB are strict anaerobes, including Bacteroidaceae, Desulfovibronaceae, Methanobacteriaceae, etc. (Nandi and Sengupta 1998), only Clostridia were found in this study. Many Clostridia are capable of producing hydrogen, including *C. acetobutylicum* (Esteso et al. 1996), *C. butylicum* (Kataoka et al. 1997), *C. butyricum* (Karube et al. 1982), *C. kluyveri* (Singh et al. 1999), and *C. pasteurianum* (McTavish 1998). At present, *Clostridium* is loosely defined as a strict anaerobe with Gram-positive cell wall structure, unable to carry out dissimilatory sulfate reduction, and capable of forming spores. It is one of the largest genera with over 100 species of diverse morphological and metabolic properties, nutritional requirements, and GC content (Stackebrandt and Rainey 1997).

The most common hydrogen-producing, facultative anaerobes are Enterobacteriaceae, followed by Lactobacillaceae and Bacillaceae (Nandi and Sengupta 1998). In this study, only Enterobacteriaceae were found. However, it is unclear whether OTU HPB-GI-4 (18.8% of the

total clones; Enterobacteriaceae) found in this study is capable of producing hydrogen. Another facultative anaerobe found in this study is in the family Streptococcaceae; and its ability to produce hydrogen is also unclear.

Most bacteria found in this study are closely related to species in the genera *Citrobacter*, *Clostridium*, or *Klebsiella*. Many bacteria in these genera are known pathogens. For instance, about 35 species of *Clostridium* are pathogenic, including *C. botulinum* and *C. difficile* (Stackebrandt and Rainey 1997); and half of these are in 16S rDNA cluster I (Fig. 1). Thus, a mixed hydrogen-producing culture is likely to be a pathogenic microbial community and thus should be handled with great care.

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