Short Communication

Unique diversity content of a thermophilic anaerobic microbial consortium that utilizes propionate in a synthetic medium

Takafumi Sugihara,¹ Hatsumi Shiratori,¹ Rieko Domoto,¹ Taku Miyake,¹ Midori Yaegashi,¹ Shohei Ayame,² Naoaki Kataoka,² Akiko Miya,² Teruhiko Beppu,¹ and Kenji Ueda^{1,*}

¹ Life Science Research Center, College of Bioresource Sciences, Nihon University, Fujisawa 252–8510, Japan

² Ebara Research Co., Ltd., Fujisawa 251–8502, Japan

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We study the application of thermophilic methane fermentation to the reduction and utilization of municipal solid wastes and non-utilized biomass. Previously, we described development and long-term operation of a thermophilic methanogenic bioreactor that digested artificial municipal solid wastes, which mainly contained shredded office paper and typical Japanese food wastes (Shiratori et al., 2006). Although the bioreactor effectively converted the substrates to methane during high performance periods, it occasionally fell into a breakdown phase due to acidification of sludge.

Here, we carried out enrichment of a propionate-degrading microbial consortium from the sludge of this bioreactor. Degradation of propionate is an important process to avoid serious fermentation breakdown; however, it is known that specific characterization of propionate-degraders is difficult due to their tight symbiotic association with other organisms in the anaerobic microbial consortia (de Bok et al., 2005; Imachi et

al., 2000). The goal of this study is to obtain a stable and culturable microbial consortium that degrades propionate and apply it to technical development for high performance reactor operation.

A methanogenic bioreactor was operated at 55°C by continuously feeding artificial municipal wastes (Shiratori et al., 2006). We occasionally observed the occurrence of fermentation breakdown due to acidification of sludge; in some cases, the methanogenic activity never recovered even after prolonged non-feeding incubation. We measured the acid content during a 125-d operation period and found that the major acids accumulated during acidification period were acetate and propionate (data not shown).

To obtain propionate-degrading microorganisms, enrichment was performed in a 100-ml top-sealed bottle flask with a working volume of 50 ml without shaking. Sludge collected from the above bioreactor was added at 30%(v/v) to M medium [a minimal medium described previously (Shiratori et al., 2006)] containing 40 mM calcium propionate as the sole carbon source and 1 mM FeCl₂ (pH 7.0). The addition of FeCl₂ was expected to support the stable growth of methanogenic Archaea (Stams et al., 1992). The culture was introduced with an N₂:H₂:CO₂=8:1:1 atmosphere and incubated at 55°C until effective gas formation was observed. The culture broth was successively inoculated into a fresh medium with the same conditions at vari-

* Address reprint requests to: Dr. Kenji Ueda, Life Science Research Center, College of Bioresource Sciences, Nihon University, 1866 Kameino, Fujisawa 252–8510, Japan.

Tel: +81–466–84–3937 Fax: +81–466–84–3935

E-mail: ueda@brs.nihon-u.ac.jp

The nucleotide sequences of 16S rRNA genes that are reported in this paper have been submitted to GenBank/EMBL/DDBJ under the accession numbers shown in Fig. 2B.

ous ratios and incubated further. Cultures that showed effective propionate metabolism and gas formation were selected and subjected to further enrichment culture. As a result of the enrichment process, we obtained a culture exhibiting a distinct propionate-digesting activity.

To isolate the microorganism responsible for the propionate-degradation in the enrichment culture, we carried out various isolation procedures including colony isolation using roll-tube lawn cultures employing several archaeal strains as symbiotic partners and selective enrichment using several antibiotics; however, we could not obtain any colony of propionate-degrading microorganisms. This was possibly due to the characteristic feature of the corresponding organism, namely dependence on complex interactions established in the microbial community. Actually, the studies on *Pelotomaculum*, the genus known for its ability to degrade propionate, have shown that cultivation of this group of bacteria is difficult even under coculture with a specific archaeal strain (de Bok et al., 2005; Imachi et al., 2002; Qiu et al., 2006). Hence, we assumed that development of an appropriate mixed (commensal) culture condition may be useful to stably maintain propionate-degrading microorganisms and their activity.

To study the stability of the propionate-metabolizing microbial consortium, the enrichment obtained above was subjected to sequencing fed batch cultivation with a periodical impact of propionate. Five milliliters of the above enrichment was inoculated into 50 ml of M medium (containing 40 mM calcium propionate) prepared in a top-sealed tube and incubated at 55°C without shaking. Forty millimolar propionate was added every 4–8 w during cultivation for 53 w. In each supply of propionate to the culture medium, the gas phase was flushed with the above anaerobic atmosphere. As shown in Fig. 1A, the culture reproducibly formed carbon dioxide and methane upon degrading propionate during the 53-w incubation. In each feeding cycle, emission of carbon dioxide occurred first, and was followed by that of methane. Propionate metabolism in every degradation cycle proceeded slowly and completed itself in ca. 6 w.

Figure 1B shows the transition of the microbial population in the continuous culture during a cycle of propionate consumption (corresponding to 18–24 w of Fig. 1A). The population was analyzed by denaturing gradient gel electrophoresis (DGGE) of partial DNA fragments of 16S rRNA gene using a D-Code system

(Bio-Rad) as described previously (Shiratori et al., 2006). The DNA fragments were amplified by PCR using the following primers: 341F (5'-CCTACGGAG-GCAGCAG; nt 341–357 by *Escherichia coli* numbering) with a 5'-terminal GC clamp (5'-CGCCCGCCGC-GCGCGGCCGGGCGGGGCGGGGGCAGCGGGGG) and Uni533R (5'-TTACCGCGGCTGCTGGCAG; nt 533–515 by *E. coli* numbering) for Bacteria, and AP5F (5'-GGCAACACGCGGGCTACAATGG; nt 1170–1191 by *Methanothermobacter thermautotrophicus* numbering) with a 5'-terminal GC clamp (5'-GCGCGCGCGCGCCC-CGCGCCCGTCCCGCCGGGCCCGCGGC) and AP7R (5'-GGCGGTGTGTGCAAGGAGCAG; nt 1352–1335 by *M. thermautotrophicus* numbering) for Archaea.

The results of DGGE analysis showed the presence of multiple archaeal and bacterial constituents in the culture. A marked change of community structure was observed in the latest phase when propionate was consumed; the archaeal population was significantly reduced in terms of its absolute content, while the bacterial population was not affected for absolute content but exhibited a distinctive change in relative content of some constituents. Similar DGGE profiles were obtained in the analysis of several other periods of operation (data not shown).

To study the diversity of content of the above propionate-metabolizing microbial consortium, a comprehensive sequencing analysis of nearly complete 16S rRNA gene was carried out. Total DNA extracted from the cellular precipitate of the culture broth was subjected to PCR using primers M85FN [5'-C(G/T)GC-TCA(G/T)TAACACGTGG; nt 85–101 by *M. thermautotrophicus* numbering] and M1350R [5'-GGCGGTGTG(C/T)GCAAGGAG; nt 1352–1332 by *M. thermautotrophicus* numbering] for Archaea, and B8F (5'-AGAGTTTGATCCTGGCTCAG; nt 8–27 by *E. coli* numbering) and 1492R [5'-GG(C/T)TACCTTGTTAC-GACTT; nt 1510–1492 by *E. coli* numbering] for Bacteria. The conditions for PCR, DNA cloning and phylogenetic analyses were described previously (Shiratori et al., 2006). Table 1 summarizes the phylogenetic affiliation of the archaeal and bacterial 16S rRNA gene sequences obtained in this study. The 16S rRNA gene fragments were also studied for their corresponding band position in the DGGE profile (designated as *a–i* in Fig. 1B) by a PCR-DGGE analysis using each cloned DNA as a template.

We succeeded in identifying three archaeal strains related to *Methanothermobacter* (phylogenetic tree is

Table 1. Phylogenetic affiliation of the members of propionate-degrading consortium.

DGGE band	Clone	Closest GenBank relative (Accession number)	Similarity % (length) ^a	Closest identified relative (Accession number)	Similarity % (length) ^a
Archaea					
<i>a</i>	EPA338	<i>Methanothermobacter wolfeii</i> (AB104845)	99 (1,234 nt)	<i>Methanothermobacter wolfeii</i> (AB104858)	99 (1,234 nt)
<i>b</i>	EPA302	Uncultured archaeon clone NAK1-a1 (DQ867048)	99 (1,234 nt)	<i>Methanothermobacter thermautotrophicus</i> strain delta H (AY196660)	95 (1,232 nt)
<i>c</i>	EPA301	Uncultured archaeon clone NAK1-a1 (DQ867048)	99 (1,234 nt)	<i>Methanothermobacter thermautotrophicus</i> strain delta H (AY196660)	96 (1,232 nt)
Bacteria					
<i>d</i>	EPE303	Uncultured bacterium clone B55_K_B_D11 (DQ887971)	96 (1,480 nt)	<i>Fervidomicrobium thiophilum</i> strain SR (EF554597)	83 (1,403 nt)
<i>e</i>	EPE124	Uncultured bacterium clone G55_D25_M_B_E12 (DQ887948)	99 (1,455 nt)	<i>Desulfotomaculum auripigmentum</i> type strain DSMZ 13352 (AJ493051)	84 (1,460 nt)
<i>e</i>	EPE313	Uncultured bacterium clone G35_D8_H_B_B06 (EF559177)	98 (1,438 nt)	<i>Clostridium tertium</i> strain DSM 2485 (Y18174)	97 (1,438 nt)
<i>f</i>	EPE306	Uncultured bacterium clone CFB-14 (AB274503)	99 (1,455 nt)	<i>Pelotomaculum isophthalicum</i> (AB232785)	94 (1,455 nt)
<i>g</i>	EPE301	Uncultured bacterium clone B55_K_B_C04 (DQ887963)	99 (1,443 nt)	<i>Thermosediminibacter oceanii</i> (X69335)	90 (1,324 nt)
<i>h</i>	EPE102	<i>Coprothermobacter</i> sp. P1 (AB162803)	99 (1,458 nt)	<i>Coprothermobacter proteolyticus</i> (X69335)	98 (1,458 nt)
<i>h</i>	EPE116	<i>Anaerobaculum mobile</i> type strain NGA (AJ243189)	91 (1,407 nt)	<i>Anaerobaculum mobile</i> type strain NGA (AJ243189)	91 (1,407 nt)
<i>h</i>	EPE305	Uncultured bacterium clone B-5 (AB233998)	98 (1,244 nt)	<i>Coprothermobacter proteolyticus</i> (X69335)	97 (1,244 nt)
<i>i</i>	EPE101	Uncultured bacterium clone G55_D25_M_B_F03 (DQ887938)	96 (1,451 nt)	<i>Thermoacetogenium phaeum</i> (AB020336)	92 (1,458 nt)

^a Length of 16S rRNA gene sequence used to calculate similarity percentage.

Fig. 1. Long-term cultivation of a propionate-metabolizing microbial consortium. (A) Carbon dioxide and methane emission during a 53-w operation. The vertical arrows indicate time points of propionic impact (40 mM). (B) Transition of gas production and microbial community structure in a cycle of propionate metabolism (corresponding to w 18–24 of the above 53-w operation). The DGGE images demonstrate the transition of community structure of Archaea (Ar) and Bacteria (Ba). Each lane corresponds to the operation date shown in the upper graph. Percentages indicate the concentration gradient of the denaturing solution supplied by the manufacturer (D-code system; Bio-Rad). Taxonomic affiliation of the organisms corresponding to band *a–i* is summarized in Table 1.

available in Fig. 2A), which corresponded to the three major DGGE bands that existed throughout the propionate metabolism (*a–c* in Fig. 1B). The strains corresponding to band *b* (EPA302) and *c* (EPA301) were distinct from the genus *Methanothermobacter* (Fig.

2A), exhibiting a low sequence identity (95–96%) to the closest identified relatives (Table 1). This suggested that these strains belong to a new taxonomic group. We failed to identify the Archaea corresponding to the DGGE bands that appeared specifically in the

Fig. 2. Unrooted tree showing phylogenetic branches of the archaeal (A) and bacterial (B) 16S rRNA genes obtained in this study. The nucleotide sequences were compared with the sequences in the GenBank/EMBL/DDBJ nucleotide sequence databases by the BLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the SEQUENCE_MATCH program using the Ribosomal Database Project (RDP) database (Cole et al., 2005). The sequences were aligned by the Clustal W program (Thompson et al., 1994). The tree, constructed by the neighbor-joining method (Saitou and Nei, 1987) using the NJ plot program (Perriere and Gouy, 1996), was based on a comparison of aligned positions of 1,200 nucleotides (excluding deleted and ambiguously aligned sites). Bootstrapping (Felsenstein, 1985) was used to estimate the reliability of the phylogenetic reconstructions; branching points supported by the neighbor-joining analysis based on 1,000 resampled datasets are indicated by closed (>80% bootstrap values) and open (>60% bootstrap values) circles. Sequences exhibiting low identity percentages (<98%) to known sequences were deposited to the GenBank/EMBL/DDBJ database. Their accession numbers are shown in the parentheses. Bar, 2% sequence divergence.

Fig. 2(A)

Fig. 2(B)

period of methane production (Fig. 1B). It is probably due to the mismatch of PCR primers used for the cloning of nearly-complete 16S rRNA gene fragments. These archaeal populations may play an important role in methane formation in this system, so the details should be characterized in future studies.

The bacterial population in the community consisted of diverged taxa of Clostridiales. Notably, most of the bacterial constituents showed low sequence similarities (<97%) to the closest identified relatives, suggesting their affiliation with new taxonomic groups. Presumably, these microbes have not yet been described partly due to the difficulty of cultivation and isolation. The assignment of DGGE bands (Fig. 1B) showed that band *d*, whose intensity increased in the latest phase, corresponded to EPE303, a member of uncultured cluster II (Fig. 2B). Band *e*, which exhibited a similar behavior to band *d*, corresponded to a member of uncultured cluster IV (EPE124) and *Clostridium* cluster I (EPE313). Band *h*, whose intensity decreased in the latest phase, corresponded to a member or a relative of *Coprothermobacter* (EPE102 and EPE305) and *Anaerobaculum* (EPE116). The unique diversity of content of the consortium consisting of many uncharacterized prokaryotes suggests the presence of unknown metabolic activities in the original methanogenic sludge.

The bacterium corresponding to EPE306 (band *f*) was a member of *Pelotomaculum*, the genus known for its involvement in propionate metabolism (de Bok et al., 2005; Imachi et al., 2002; Qiu et al., 2006). The 16S rRNA gene sequences almost identical to that of EPE306 have been obtained in propionate-degrading enrichments (Imachi et al., 2006) and a thermophilic packed-bed bioreactor degrading garbage (Sasaki et al., 2007), although the corresponding bacterium has not yet been isolated. Hence, it is likely that this unknown *Pelotomaculum* sp. is involved in propionate metabolism in various digestion systems, and that it comprises a complex association with other member of the community, which makes it difficult to isolate the organism.

The propionate metabolism reproducibly observed during the long-term cultivation suggests that the microbial consortium obtained in this study is stable and suitable for cultivation. We think it worthwhile to augment the consortium to the original bioreactor to study its effect on propionic content. The culturability in a synthetic medium will be useful in preparation of the

augmentation source. The culture appears also useful in terms of storage; the propionate-metabolizing activity of the consortium was successfully recovered after freezing storage at -80°C for 2 months (our unpublished observation). We also anticipate that the detailed basic characterization of this consortium will facilitate understanding the metabolic activity of unknown prokaryotes as well as the significance of their community structuring in anaerobic digestion systems.

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