Diversity and physiology of syntrophic substrate-oxidizing anaerobes in methanogenic ecosystems

メタン生成エコシステムにおける嫌気共生細菌の多様性と生態

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Introduction

Under methanogenic conditions, degradation of complex organic substances is carried out by cooperation of different trophic groups of anaerobic microorganisms. In this type of microbial ecosystems, fatty acids and other compounds, such as ethanol and benzoate, are frequently formed as intermediates. Since such intermediates frequently accumulate in this type of ecosystems, microbes involved in the degradation of these intermediates are important. The degradation of intermediates is generally carried out by substrate oxidizing, proton-reducing microbes (syntrophic bacteria), and hydrogenotrophic microbes. Intermediates are converted by syntrophic bacteria to form acetate, hydrogen and CO_2 only when the hydrogen partial pressure is kept extremely low by hydrogenotrophic microbes. The degradation of intermediates is generally carried out by substrate oxidizing, proton-reducing microbes (syntrophic bacteria), and hydrogenotrophic microbes. Intermediates are converted by syntrophic bacteria to form acetate, hydrogen and CO_2 only when the hydrogen partial pressure is kept extremely low by hydrogenotrophic microbes [1,2,3,4,5].

Despite their significant metabolic functions in methanogenic ecosystem, information on the physiological characteristics and phylogenetic diversity of such organisms was limited, especially due to the fastidious characteristics of the syntrophic microbes, such as slow growth, low growth yield and syntrophic association with hydrogenotrophic microbes. In this study, the diversity and physiology of syntrophic substrate-oxidizing anaerobes were studied by applying conventional cultivation techniques combined with rRNA-based molecular approaches [6]

Material and methods

For cultivation of anaerobic, syntrophic bacteria, two thermophilic (55°C) anaerobic digested sludges, one mesophilic (35°C), anaerobic digested sludge, a mesophilic, anaerobic granular sludge and rice paddy soil were used as inoculum for primary enrichments, using ethanol (10 mM), benzoate (5 mM) or propionate (20 mM) as the sole carbon source. DNA extraction from enrichment cultures was performed by bead-beating method. For 16S rDNA-based clone analysis, a bacterial universal primer set was used. Fluorescence in situ hybridization (FISH) was carried out according to the method of Amann et al. [7].

Result and discussion

1. Enrichment of anaerobic, syntrophic bacteria

For enrichment of anaerobic, syntrophic bacteria, various types of anaerobic, environmental samples were used for primary enrichment using ethanol, benzoate or propionate as the sole carbon source. The growth of microbes in these cultures was observed after one to three months of incubation. After 5 times successive transfers, these cultures produced methane along with substrate depletion. All enrichment cultures contained F_{420}-autofluorescent microbes and several morphologically distinct microbes. From this observation, it was suggested that the substrate-degadation in almost all of the enrichment cultures was carried out by the syntrophic bacteria and methanogens syntrophically.

Table 1. Products detected during methanogenic process in enrichment cultures

Sludge	Substrate	Substrate Converted (mM)	Products				Elec. Rec. (%)
			Acetate(mM)	Methane (mM)	Propionate (mM)	Butyrate (mM)	
1	Ethanol	9.5	5.59	5.38	0.74	0	99.01
2	Ethanol	10	6.78	4.31	0.00	0	85.03
3	Ethanol	11	9.18	4.00	0.00	0	91.83
4	Ethanol	9.3	6.63	4.80	0.00	0	94.24
5	Ethanol	9.7	6.38	3.99	0.00	0	81.98
1	Benzoate	4.5	0.26	14.76	0.00	0	89.01
2	Benzoate	2.02	5.88	1.33	0.13	0	98.34
3	Benzoate	4.5	6.67	3.62	1.11	1.11	72.47
1	Propionate	15.3	0.41	18.85	0.00	0	71.87
2	Propionate	8.1	0.00	10.40	0.00	0	73.25
3	Propionate	3.7	2.42	0.17	0.00	0	39.98
5	Propionate	8.44	0.87	13.85	0.00	0	99.68

Notes: 1. Granular sludge, artificial organic wastewater treatment (37^{0}C)
2. Digested sludge, sewage treatment plant, Nagaoka (37^{0}C)
3. Rice paddy soil, Nagaoka (37^{0}C)
4. Digested sludge, sewage treatment plant, Osaka (55^{0}C)
5. Digested sludge, municipal solid waste treatment plant, Joetsu (55^{0}C)

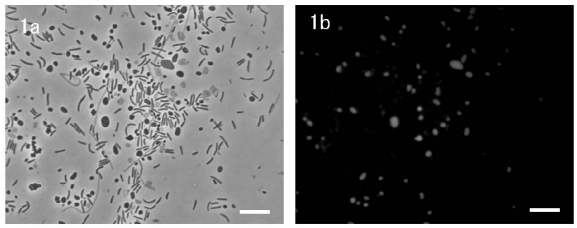

Fig.1. Phase contrast (1a) and fluorescence-F_{420} (1b) micrograph of mesophilic benzoate enrichment culture at same field, inoculated with digested sewage sludge, Nagaoka/ Bar : 10 µm.

2. 16S rRNA-based analysis for syntrophic bacteria in enrichment cultures

To identify bacteria involved in syntrophic substrate-degradation, 16S rDNA-based analysis was primary performed for all enrichment cultures. Ten rDNA clones were randomly selected from

Table 2. Bacterial 16S rDNA libraries of enrichmnet culturtes

	Sludge	Number of clones /total clones	Closest organism	Similarity (%)
ETHANOL	1	6 / 10	*Desulfovibrio desulfuricans*	97
		2 / 10	*Geobacter bremensis*	95
		2 / 10	*Acidaminococcus sp (Firmicutes, Clostridia)*	90
	2	8 / 10	*Geobacter* sp. , strain CdA-3	95
		1 / 10	Uncultured eubacterium WCHB1-29	90
		1 / 10	*Anaerofilum agile (Firmicutes; Clostridia)*	99
	3	7 / 10	*Geobacter* sp. JW-3 16S	95
		2 / 10	Proteobacterium KCB90	99
		1 / 10	*Desulfovibrio sulfodismutans*	97
	4	10 / 10	*Thermodesulfovibrio* sp	91
	5	10 / 10	Uncultured clone (strain JE)	91
BENZOATE	1	10 / 10	*Syntrophus acidotrophicus*	98
		5 / 10	Uncultured clone (= BD strain)	88
	2	4 / 10	*Clostridium quercicolum*	92
		1 / 10	*Syntrophus acidotrophicus*	96
	3	9 / 10	*Sporotomaculum syntrophicum*	99
		1 / 10	*Desulfotomaculum guttoideum*	98
	4		NOT YET	
	5		NOT YET	
PROPIONATE	1	8 / 10	*Desulfobulbus elongatus*	91
		2 / 10	*Syntrophobacter pfennigii*	90
	2	9 / 10	Anaerobic bacterium strain 7 (*Pelotomaculum* s	92
		1 / 10	*Coryobacterium glomerans*	
	3	10 / 10	*Sporomusa* sp. DR6 *(Firmicutes)*	99
	4		NOT YET	
	5		NOT YET	

Notes: 1. Granular sludge, artificial organic wastewater treatment (37°C)
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5. Digested sludge, municipal solid waste treatment plant, Joetsu (55°C)

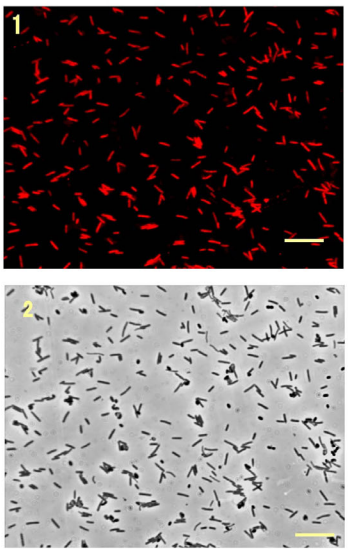

Fig. 2. Fluorescence in situ hybridization of mesophilic benzoate enrichment. Cells were hybridized with Cy3-labeled BD202 probe. Fluorescence (1) and phase contrast (2) micrograph of an identical field of mesophilic benzoate enrichment culture. Bar 10 µm.

each enrichment culture, and their sequences were determined. Among the predominant clones recovered from each enrichment culture, some clones showed close relation with known bacteria to date as syntroph, such as *Desulfovibrio* sp. Nonetheless, several clones seemed to indicate novel bacterial lineages that have never cultivated and isolated so far, such as clones related with the genus *Geobacter* in two mesophilic ethanol enrichments, clones representing a deeply branched lineage of the phylum *Firmicutes* in a thermophilic ethanol enrichment culture, and clones related with the genus *Desulfobulbus* in a mesophilic propionate degrading anaerobes.

To determine whether the dominant clones were derived from the dominant microbes in enrichment cultures, specific DNA probes were designed and applied for the cultures in fluorescence in *situ* hybridization analyses. This resulted in the detection of a number of DNA probe-reacted cells in all the cultures, suggesting the probe-positive cells were the dominant microbes in the cultures [8,9]. Table 3 shows the oligonucleotide probes used in this study, included new probes design of BD202, BD1009 and RPC6.

Both of probe BD were applied to hybridize newly identified bacteria, obtained from benzoate degrading culture (mesophilic) and ethanol degrading culture (thermophilic). While RPC6 was employed to detect a new bacteria in mesophilic propionate degrading culture

Table 3. The 16S rRNA-targeted oligonucleotide probes used in this study

Probe name	Probe sequence (5' to 3')	Target microbe	Reference
EUB338	GCTGCCTCCCGTAGGAGT	*Bacteria*	Aman et al., 1990
EUB338-II	GCAGCCACCCGTAGGTGT	*Bacteria*	Daims et al., 1999
EUB338-III	GCTGCCACCCGTAGGTGT	*Bacteria*	Daims et al., 1999
D687	TACGGATTTCACTCCT	*Desulfovibrio* sp	Devereux et al., 1992
Geob420	GACAGAGCTTTACGACCCG	*Geobacter* sp	Small et al., 2001
TDV1011	CCCCTAAGGTCGTCCCCCTT	*Thermodesulfovibrio* sp	Sekiguchi et al., 1999
Ih 820	ACCTCCTACACCTAGCACCC	*Pelotomaculum* sp	Imachi et al., 2001
DEM1164	CCTTCCTCCGTTTTGTCA	*Sporotomaculum* sp	Stubner &Meuser, 2001
BD1009	GTCGATTCCGTTTCCGGT	Strain BD	This study
BD202	CATGACCGCTAAATCGCT	Strain JE	This study
RP452	GTCTTTCTCCTTAAGGCC	Mesophilic propionate enrichment culture	This study

3. Attempts at isolation of syntrophic substrate-degrading bacteria

Phylogenetic analysis and in situ hybridization experiments identified the dominant, presumably syntrophic microorganisms in each enrichment culture. To isolate such newly identified bacteria, a molecular-directed isolation strategy was employed; it assumes the physiological properties of novel identified bacteria based on the information obtained from phylogenetic analysis.

3.1. Uncultured bacteria, phylum Firmicutes

The phylogenetic tree of newly identified bacteria obtained from mesophilic benzoate enrichment culture (designed as strain BD) and thermophilic ethanol enrichment culture are illustrated in Fig. 3.

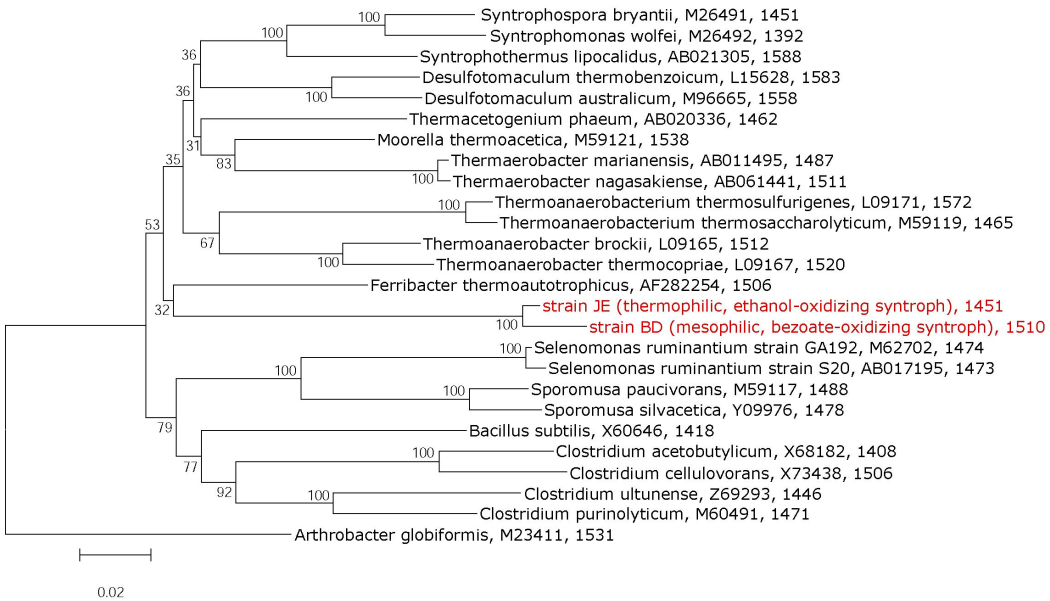

Fig. 3. Phylogenetic tree of strain BD and JE and related organism based on the distance matrix analysis of 16S rDNA sequence. The scale bar represents the number at the nodes are the bootstrap values obtained with 100 resampling analysis

These bacteria represent the deeply branched lineage of phylum *Firmicutes*. Both of these bacteria have been isolated in pure culture (Fig. 4).

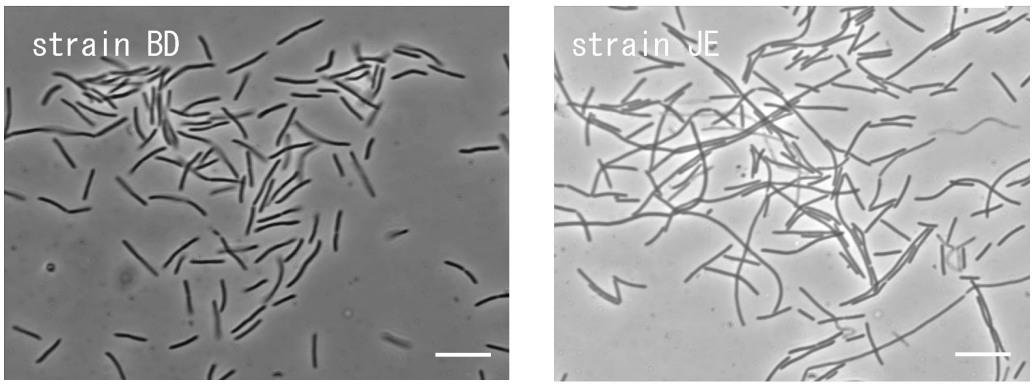

Fig.4. Micrograph of pure, new identified bacteria, strain BD and strain JE, members of phylum *Firmicutes*. Strain BD and JE were grown on crotonate 5 mM + yeast extract 0.02% and sucrose 20 mM + yeast extrtact 0.01%, respectively. Bar represent10 µm

3.2. *Thermodesulfovibrio* sp-like bacterium

The phylogenetic tree of *Thermodesulfovibrio* sp-like, isolated from thermophilic ethanol enrichment culture shown at Fig. 6. Pure culture was obtained at substrate of formate, contained acetate, sulfate and yeast extract (Fig. 5).

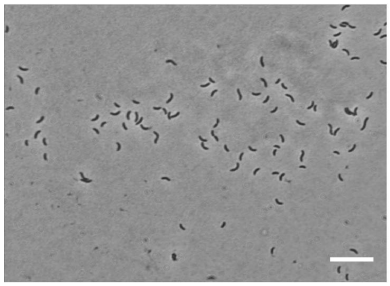

Fig.5. Micrograph of strain OE (*Thermodesulfovibrio* sp-like). Cells were grown on formate 15 mM + acetate 2mM + sulfate 20 mM + yeast extract 0.02% and sucrose 20 mM + yeast extrtact 0.02%. Bar represent10 µm.

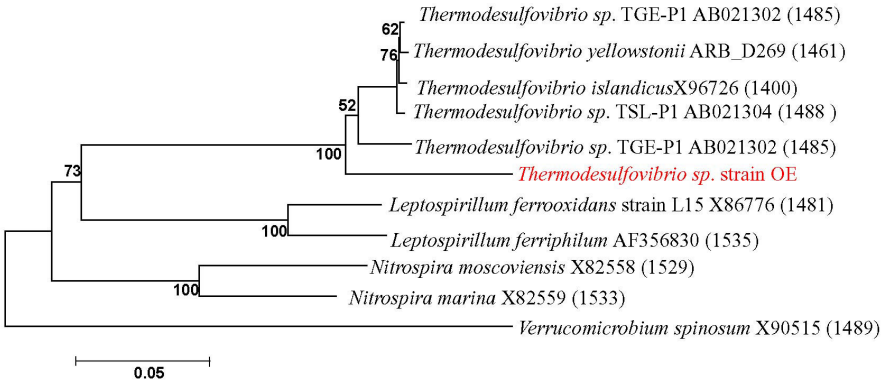

Fig. 6. Phylogenetic tree of *Thermodesulfovibrio* strain OE and related organism based on the distance matrix analysis of 16S rDNA sequence. The scale bar represents the number at the nodes are the bootstrap values obtained with 100 resampling analysis

3.3. *Geobacter* sp-like bacteria

Geobacter sp-like were obtained from two mesophilic ethanol enrichment cultures, designed as clone NE1 and FE1. Figure 8 illustrates the phylogenetic of such bacteria. Both of them have been cultivated as well, however, isolation have not been succeed yet.

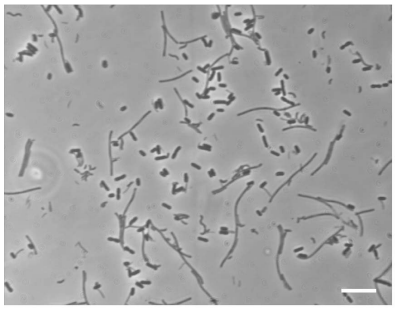

Fig.7. Micrograph of *Geobacter* sp-like bacterium (clone NE1), mesophilic ethanol enrichment culture. 0.02%. Bar represent10 µm.

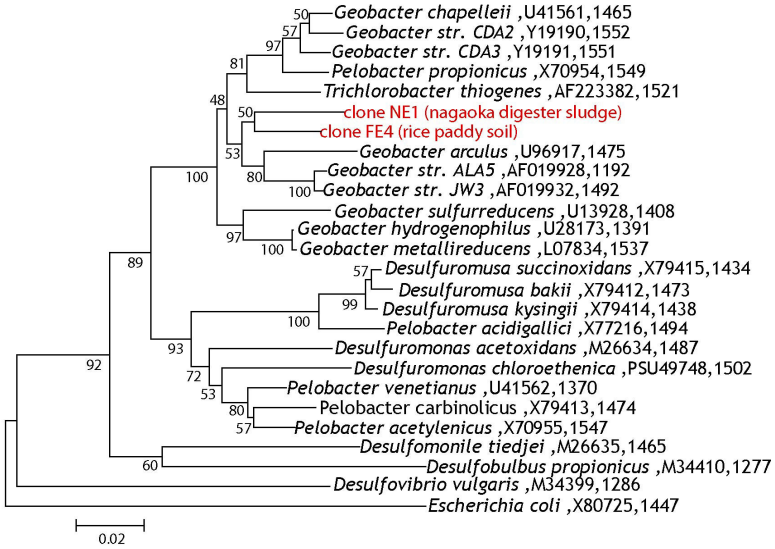

Fig. 8. Phylogenetic tree of *Geobacter* sp-like (clone NE1) and related organism based on the distance matrix analysis of 16S rDNA sequence. The scale bar represents the number at the nodes are the bootstrap values obtained with 100 resampling analysis

3.4. *Desulfobulbus* sp-like

A stable mesophilic propionate degrading culture have been cultivated well after five times successive transfer. Based on 16S rDNA cone analysis, this bacterium was indicated as a new bacterium, closely related to *Desulfobulbus* sp. Phylogenetic tree of this bacterium have been constructed in Fig. 10.

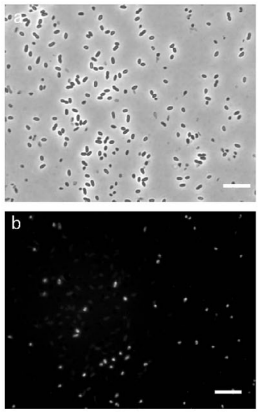

Fig.9. Phase contrast (a) and fluorescence-F_{420} (b) micrograph of mesophilic propionate enrichment culture (RP) at same field. Bar : 10 µm.

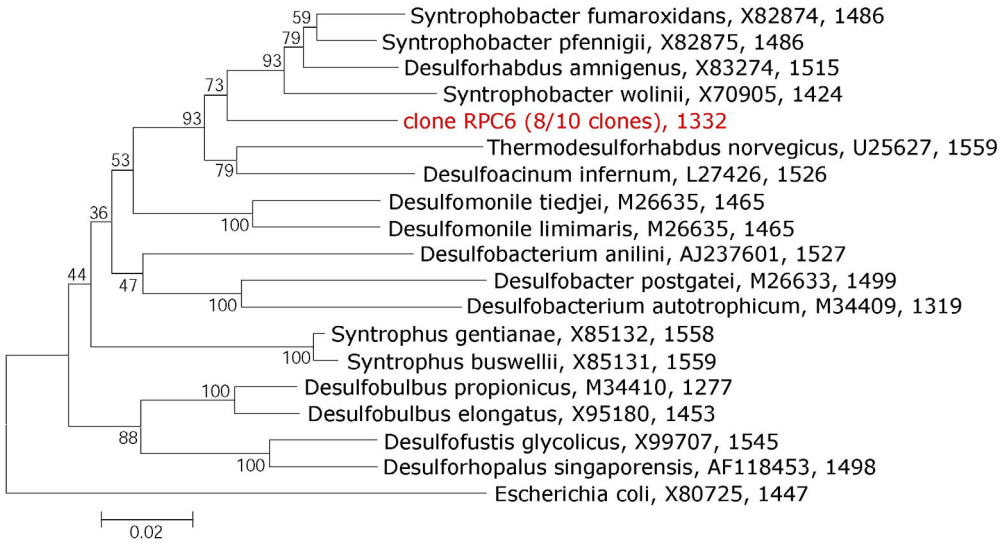

Fig. 10. Phylogenetic tree of clone RPC6 and related organism based on the distance matrix analysis of 16S rDNA sequence. The scale bar represents the number at the nodes are the bootstrap values obtained with 100 resampling analysis

Nevertheless, such bacterium have not been isolated in pure culture yet, although several attempts have been conducted.

Conclusion

This study strongly suggested that the strategy employing conventional techniques combined with 16S rRNA-based approaches is advantageous to determine the diversity of recalcitrant microbes like syntrophic microorganisms and to attempt at subsequent isolation of targeted cells. The known syntrophic microbes obtained from this study were *Desulfovibri*o sp, *Syntrophus* sp and *Sporotomaculum* sp, obtained from mesophilic cultivation of ethanol and benzoate enrichment cultures, respectively. Instead of those, several clones represented the bacterial that have never cultivated and isolated so far as syntrophic microbes. The such newly identidied bacteria obtained from benzoate (mesophilic), propionate (mesophilic) and ethanol (mesophilic and thermophilic) enrichment cultures.

To isolate such newly identified bacteria, a molecular-directed isolation strategy was applied. Through this approach, two novel syntrophic, ethanol-oxidizing bacteria, designed as strain JE and OE; and a novel benzoate-degrading bacterium, designed as strain BD were successfully isolated. Additionally, two bacteria presumably new syntrophic microbes were cultivated as well. They were *Geobacter* sp-like (from mesophilic ethanol cultures) and *Desulfobulbus* sp-like (mesophilic propionate culture).

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