Behavior of cellulose-degrading bacteria in thermophilic anaerobic digestion process

K. Syutsubo*, Y. Nagaya, S. Sakai*** and A. Miya****

*National Institute for Environmental Studies, Water and Soil Environment Division, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan (E-mail: *stubo@nies.go.jp*)

**Ebara Corp., Technologies, R. & D. Division, 4-2-1 Honfujisawa, Fujisawa, Kanagawa 251-8502, Japan

***Department of Environmental Systems Engineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan

Abstract Previously, we found that the newly isolated *Clostridium* sp. strain JC3 became the dominant cellulose-degrading bacterium in thermophilic methanogenic sludge. In the present study, the behavior of strain JC3 in the thermophilic anaerobic digestion process was investigated quantitatively by molecular biological techniques. A cellulose-degrading experiment was conducted at 55 °C with a 9.5 L of anaerobic baffled reactor having three compartments (Nos. 1, 2, 3). Over 80% of the COD input was converted into methane when 2.5 kgCOD m^{-3} d^{-1} was loaded for an HRT of 27 days. A FISH probe specific for strain JC3 was applied to sludge samples harvested from the baffled reactor. Consequently, the ratio of JC3 cells to DAPI-stained cells increased from below 0.5% (undetectable) to 9.4% (compartment 1), 13.1% (compartment 2) and 21.6% (compartment 3) at day 84 (2.5 kgCOD m^{-3} d^{-1}). The strain JC3 cell numbers determined by FISH correlated closely with the cellulose-degrading methanogenic activities of retained sludge. A specific primer set targeting the cellulase gene (*cellobiohydrolaseA*: *cbhA*) of strain JC3 was designed and applied to digested sludge for treating solid waste such as coffee grounds, wastepaper, garbage, cellulose and so on. The strain JC3 cell numbers determined by quantitative PCR correlated closely with the cellulose-sludge loading of the thermophilic digester. Strain JC3 is thus important in the anaerobic hydrolysis of cellulose in thermophilic anaerobic digestion processes.

Keywords Cellulose; *Clostridium*; microbial community structure; quantitative population analysis; thermophilic anaerobic digestion

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Introduction

Anaerobic digestion has become a major technology in the treatment of solid wastes, such as sludge, municipal refuse (garbage, paper) and so on. These types of waste contain a variety of organic compounds, of which cellulose is the most prominent. It is also rather difficult to degrade biologically (Schwarz, 2001). In order to develop appropriate technology to enhance the biodegradation of cellulose in the anaerobic digestion process, it is necessary to accumulate basic information on methanogenic microbial communities that degrade cellulose-containing wastes.

In our previous study, we found that the biodegradation of cellulose was far more stimulated in thermophilic conditions (55 °C) than in mesophilic conditions. Furthermore, as a result of microbial community analysis of thermophilic cellulose enrichment cultures, novel *Clostridium* strains (strain JC3 and its relatives, Syutsubo *et al.*, 2003, Nagaya *et al.*, in preparation) became dominant as major cellulose degrading bacteria. In the present study, the behavior of strain JC3 in the thermophilic anaerobic digestion process was investigated quantitatively by molecular biological techniques (fluorescence *in situ* hybridization, quantitative PCR) to elucidate the ecological significance of strain JC3 when used for treating cellulose.

Materials and methods

Cellulose-degrading experiment

A cellulose-degrading experiment was conducted at 55 °C using an anaerobic baffled reactor with total volume of 9.5 L. This reactor has 3 compartments (Nos. 1, 2, 3) of equal volume. The sludge retained in each compartment was mixed continuously by agitator. In this study, thermophilically (55 °C) digested sludge harvested from a full-scale digester receiving night soil/septic tank sludge and garbage was used as the inoculum (Yoneyama and Takeno, 2002). The feed solution was composed of cellulose powder (40 to 100 mesh, ADVANTEC) and yeast extract at a COD ratio of 95:5. Feed was provided once a day. A continuous experiment was started at a COD volumetric loading of 1.1 kgCOD m^{-3} d^{-1} with an influent COD strength of 44 gCOD l^{-1} and a hydraulic retention time (HRT) of 40 days. The volumetric COD loading increased stepwise by reducing HRT, but also by increasing the concentration of influent COD. The proportions of basal minerals and trace elements were the same as those in our previous study (Syutsubo *et al.*, 2001b).

Analyses

The composition of the generated biogas was analyzed with a TCD gas chromatograph (G.L. Science, model GC322) equipped with a stainless-steel column of active carbon (30/60 mesh). Volatile fatty acids (VFA) were determined on a high-performance liquid chromatograph (HPLC, Shodex) equipped with a differential thermal analyzer. The methanogenic activity of retained sludge was determined at 55 °C by the method described in previous studies (Syutsubo *et al.*, 1997, Syutsubo *et al.*, 2003). The test substrates used were acetate, H_2/CO_2 (80%:20%, v/v), cellulose. The microbial community structure of retained sludge was determined by domain Bacterial 16S rDNA-targeted PCR-DGGE analysis (Muyzer *et al.*, 1993, Syutsubo *et al.*, 2003).

Enumeration of cellulose degrading bacteria (strain JC3)

Enumeration of *Clostridium* sp. strain JC3 cells in the retained sludge was conducted by fluorescence *in situ* hybridization (FISH) with a specific oligonucleotide probe (JC3-588, *E. coli* 16S rDNA, positions 588 to 604) targeting the 16S rRNA of *Clostridium* sp. strain JC3. FISH was performed as described previously (Syutsubo *et al.*, 2001a, 2001b) at 46 °C for 3 hr with a hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 7.2], 0.01% SDS) containing 10% (v/v) of formamide.

For several kinds of digested sludge that receive cellulose-containing wastes (coffee grounds, wastepaper etc.), the quantification of strain JC3 cells was conducted by competitive PCR. CelK14F (JC3 *cbhA* position: 719–741) and CelK14R (JC3 *cbhA* position: 845–869) primers were selected to amplify a 150 bp fragment of the *cbhA* of strain JC3. As an internal standard, we used a competitor prepared with a Competitive DNA Construction Kit (TaKaRa). The PCR conditions used were as follows: 10 min at 94 °C; 35 cycles at 95 °C, 55 °C and 72 °C for 1 min each; and finally 10 min at 72 °C.

The nucleotide sequences of 16S rDNA and cellulase gene (*cellobiohydrolaseA*) of the isolated bacterium *Clostridium* sp. strain JC3 have been submitted to the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB093546 and AB093547.

Results and discussion

Cellulose-degrading experiment

A cellulose-degrading experiment was conducted using an anaerobic baffled reactor at 55 °C for over 90 days. A mixture of cellulose and yeast extract (95:5, in the ratio of

the total COD) was used as feed. The COD volumetric loading increased stepwise from 1.1 kgCOD $m^{-3} d^{-1}$ to 2.5 kgCOD $m^{-3} d^{-1}$ by both reducing the HRT and increasing the influent COD concentration. As a result, the COD sludge loading increased from 0.13 gCOD $gVSS^{-1} d^{-1}$ (Day 0) to 1.38 gCOD $gVSS^{-1} d^{-1}$ (Day 72, 2.5 kgCOD m^{-3}-d^{-1}). More than 80% of the input COD was converted to methane when the COD loading and HRT were 2.5 kgCOD $m^{-3} d^{-1}$ and 27 days, respectively. The effluent VFA concentration was kept comparatively low level throughout. This phenomenon indicated that the hydrolysis of cellulose is probably a rate-limiting step in this process.

The methane production (biodegradation of cellulose) from each compartment of the reactor was almost uniform until COD loading of 1.66 kgCOD $m^{-3} d^{-1}$ (Figure 1). However, when the COD loading was increased to 2.5 kgCOD $m^{-3} d^{-1}$, methane production from the last compartment (No. 3) was clearly accelerated. The cellulose fibers used as feed were partially degraded and were divided (enlarged surface area) between compartments 1 and 2. The divided fiber load, which contained large amounts of cellulose-degrading bacteria (Figure 3) reached the final compartment (No. 3), indicating that biodegradation of cellulose may be accelerated in compartment No. 3 under conditions of high COD loading.

The methane-producing activity of retained sludge harvested from each compartment was determined at day 35 (1.66 kgCOD $m^{-3} d^{-1}$) and day 84 (2.5 kgCOD $m^{-3} d^{-1}$) (Table 1). These methanogenic activities were almost uniform in all compartments for all substrates at day 35. Later, on day 84, cellulose-fed activity reached 0.64, 0.68 and 1.4 gCOD $gVSS^{-1} d^{-1}$ in sludge samples harvested from compartments 1, 2 and 3, respectively. This result demonstrates that the cellulose-degrading potential of retained sludge was greatly promoted in sludge obtained from compartment 3 at a COD loading of 2.5 kgCOD $m^{-3} d^{-1}$. These changes correspond to the pattern of methane production from each compartment of the baffled reactor (Figure 1).

Microbial structure analysis (enumeration of cellulose-degradaing strain JC3)

The microbial community structures of sludge samples were analyzed by domain Bacterial 16S rDNA-targeted PCR-DGGE analysis. As a result of the DGGE analysis, prominent growth of the thermophilic cellulose-degrading bacterium, *Clostridium* sp. strain JC3, was observed (data not shown). *Clostridium* sp. strain JC3 was isolated from the thermophilic cellulose enrichment culture by a combination of most probable number (MPN) series dilution and the roll-tube method. The optimum growth temperature and pH were 55 °C and 7.0, respectively. This strain can grow on cellulose or cellobiose. As

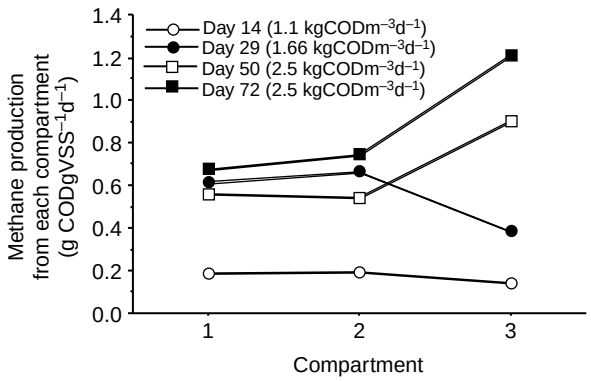

Figure 1 Profiles of methane production during progress through the baffled reactor

Table 1 Methanogenic activities of retained sludge determined at 55 °C, standard deviation is given between parentheses

Sample	Methane producing activity ($gCOD\,gVSS^{-1}\,d^{-1}$)		
	Acetate	H_2/CO_2	Cellulose
Day 0, seed	0.003 (0.0006)	0.91 (0.06)	0.003 (0.0005)
Day 35 1.66 $kgCOD\,m^{-3}\,d^{-1}$	0.25 (0.016)	3.07 (0.05)	0.46 (0.031)
Comp. No.1	0.29 (0.005)	3.01 (0.29)	0.47 (0.009)
Comp. No.2	0.28 (0.0001)	3.05 (0.026)	0.44 (0.011)
Comp. No.3			
Day 84 2.5 $kgCOD\,m^{-3}\,d^{-1}$	1.02 (0.0096)	1.92 (0.09)	0.64 (0.053)
Comp. No.1	1.15 (0.077)	1.63 (0.18)	0.68 (0.001)
Comp. No.2	1.51 (0.11)	0.92 (0.16)	1.38 (0.039)
Comp. No.3			

a result of cellulose-degradation, strain JC3 produced sugars, ethanol, acetate, lactate and hydrogen (Nagaya *et al.*, in preparation).

A FISH probe specific for strain JC3 (JC3-588) was designed and was applied to retained sludge to determine the population of strain JC3. As a result of FISH analysis, the ratio of JC3 cells to DAPI-stained total cells increased from less than 0.5% (day 0, undetectable level) to 9.4% (compartment No. 1), 13.1% (No. 2) and 21.6% (No. 3) at day 84 (2.5 $kgCOD\,m^{-3}\,d^{-1}$) (Figure 2).

The presence ratio of strain JC3 determined by FISH was closely correlated with the cellulose-degrading methanogenic activity of sludge samples obtained from each compartment of the baffled reactor (Table 1). This result indicates that strain JC3 plays an important role in the anaerobic biodegradation of cellulose in this thermophilic methanogenic consortium.

Furthermore, strain JC3 cells specifically were attached to, and grew on, the cellulose fibers (Figure 3). Adhesion of cellulose-degrading bacteria to the cellulose fibers was also observed in other research (Noike *et al.*, 1985; Burrell *et al.*, 2004).

In order to investigate the distribution and ecological significance of cellulose-degrading strain JC3, a specific primer set which targets the cellulase-gene (*cellobiohydrolaseA*: *cbhA*) of strain JC3 was devised and applied to the digested sludge used to treat several kinds of solid waste, such as coffee grounds, wastepaper, garbage, cellulose and so on. Previously, we had confirmed that the copy number of the *cbhA* gene was proportional to the cell number of strain JC3 in the sludge sample.

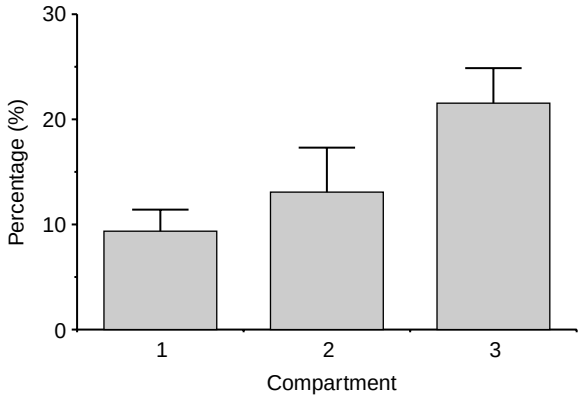

Figure 2 Ratios of populations of strain JC3 cells present in the retained sludge harvested from the compartments of the baffled reactor at 2.5 kg $COD\,m^{-3}\,d^{-1}$

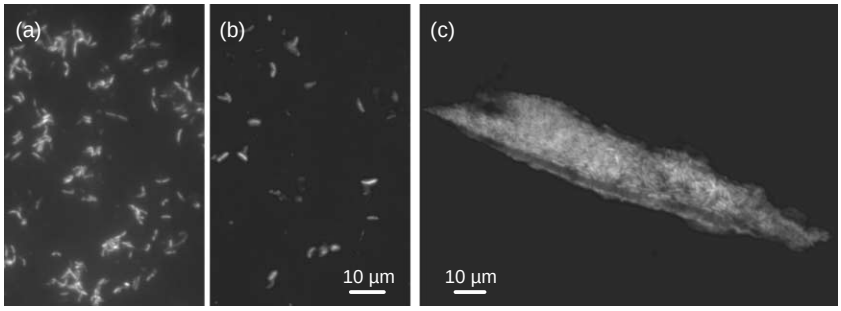

Figure 3 Fluorescence micrographs of cellulose-degrading sludge which was sampled from the baffled reactor: (a) all DAPI-stained cells, (b) JC3 cells hybridized with a specific probe JC3-588, (c) probe-hybridized JC3 cells presented in abundance in cellulose fibers

The *cbhA* gene of strain JC3 was clearly detected in thermophilic digested sludge. The copy numbers of *cbhA* were 2.52×10^{10} copies $gVSS^{-1}$, 8.65×10^{10} copies $gVSS^{-1}$ and 1.60×10^{13} copies $gVSS^{-1}$ in sludge used to treat coffee grounds, wastepaper and cellulose, respectively. Cellulose-degrading potential of thermophilic methanogenic sludge increased clearly according to the accumulation of strain JC3 in the sludge. However, *cbhA* was not detected (less than 10^7 copies g VSS^{-1}) in mesophilic or ambient anaerobic sludge samples. The copy numbers of *cbhA* gene, synonymous with the cell numbers of strain JC3, and determined by quantitative PCR, were closely correlated with the cellulose-sludge loading of the thermophilic digester (Figure 4). This indicates that determination of the cell numbers of strain JC3 in thermophilic anaerobic sludge may predict the cellulose-degrading potential of the sludge sample.

Conclusions

From these observations, strain JC3 has an important role in the hydrolysis of cellulose in methanogenic consortia, especially in thermophilic conditions. Furthermore, monitoring and controlling the strain JC3 were important for stabilization and improvement of cellulose biodegradation in the thermophilic anaerobic digestion process. Currently, we are

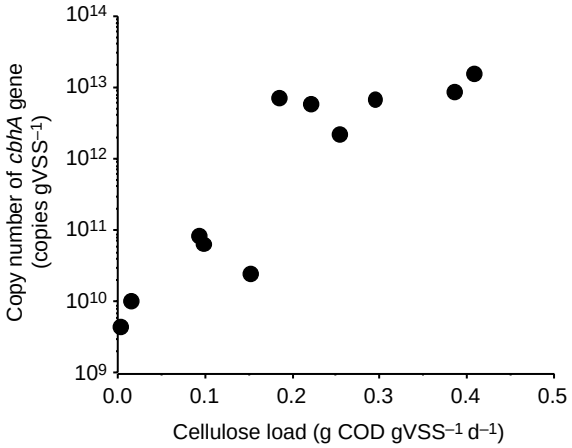

Figure 4 Correlation between cellulose sludge loading and copy number of *cbhA* gene with respect to thermophilic digested sludge used for treating several kinds of solid wastes

investigating the possibility of using the strain JC3 for shortening the start-up period of the digester for the treatment of cellulose-containing wastes.

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