Journal of Bioscience and Bioengineering
VOL. 112 No. 5, 469–472, 2011

www.elsevier.com/locate/jbiosc

Syntrophic degradation of proteinaceous materials by the thermophilic strains *Coprothermobacter proteolyticus* and *Methanothermobacter thermautotrophicus*

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Received 30 May 2011; accepted 4 July 2011
Available online 29 July 2011

Protein is a major component of organic solid wastes, and therefore, it is necessary to further elucidate thermophilic protein degradation process. The effects of hydrogenotrophic methanogens on protein degradation were investigated using the proteolytic bacterial strain CT-1 that was isolated from a methanogenic thermophilic (55°C) packed-bed reactor degrading artificial garbage slurry. Strain CT-1 was closely related to *Coprothermobacter proteolyticus*, which is frequently found in methanogenic reactors degrading organic solid wastes. Strain CT-1 was cultivated in the absence or presence of *Methanothermobacter thermautotrophicus* by using 3 kinds of proteinaceous substrates. Degradation rates of casein, gelatin, and bovine serum albumin were higher in co-cultures than in monocultures. Strain CT-1 showed faster growth in co-cultures than in monocultures. *M. thermautotrophicus* comprised 5.5–6.0% of the total cells in co-culture. Increased production of ammonia and acetate was observed in co-cultures than in monocultures, suggesting that addition of *M. thermautotrophicus* increases the products of protein degradation. Hydrogen produced in the monocultures was converted to methane in co-cultures. These results suggest that thermophilic proteolytic bacteria find it favorable to syntrophically degrade protein in a methanogenic environment, and that it is important to retain hydrogen-scavenging methanogens within the reactor.

[**Keywords:** Protein; Methane fermentation; Proteolytic bacteria; Hydrogenotrophic methanogen; Co-culture]

Anaerobic treatment is an effective technology for processing organic solid wastes (1). Protein is one of the main components of organic waste, in addition to carbohydrates (2). Organic materials such as whey, cheese, fish, and certain vegetables contain significant amounts of protein (2,3). However, the majority of investigations on anaerobic treatments focused on carbohydrate degradation (3).

In methanogenic reactors, the proteins are hydrolyzed to peptides and amino acids, which are then degraded to volatile fatty acids (VFAs), ammonium, and hydrogen (4,5). VFAs are converted into acetate and/or H_2/CO_2, both of which are converted to methane. Amino acid degradation is classified into 2 types; pairs of amino acid are degraded through the Stickland reaction or single amino acids are syntrophically fermented in the presence of hydrogen-utilizing microorganisms (3). In the syntrophic degradation of some amino acids, anaerobic oxidation reactions with a positive Gibbs free energy change are possible when the products are removed by methanogens (6). Most anaerobic amino acid degrading bacteria are affiliated with the phylum *Firmicutes* (2,3). For example, *Gelria glutamica* can grow on glutamate and proline in co-culture with the hydrogenotrophic methanogen (7). The possibility that a syntrophic reaction would overcome Stickland reaction in protein degradation is suggested in the mesophilic completely stirred tank reactor wherein enough hydrogen-utilizing methanogens exist (2). Lately, anaerobic processing under thermophilic conditions is a prevalent method, because it is a faster reaction, with higher gas production, and higher destruction of pathogens than that under mesophilic conditions (8). However, only a few thermophilic proteolytic bacteria have been isolated and described as being capable of conducting syntrophic degradation of proteins (9).

In this study, we have isolated a thermophilic proteolytic anaerobe, *Coprothermobacter proteolyticus*-related species (10,11), from a packed-bed reactor. Microorganisms related to this isolated organism are often observed in methanogenic reactors degrading organic solid wastes and affiliated with family *Thermodesulfobiaceae*, which is differently branched from families including most amino acid degrading bacteria in the phylum *Firmicutes*. Reportedly *C. proteolyticus* degrades casein and gelatin, and hydrogen is one of the major products (10,11). Although syntrophic degradation of amino acids was not reported about this microorganism, there is the possibility that this degrades protein syntrophically with hydrogen-consuming microorganisms. The aim of this paper is to describe protein degradation in thermophilic reactors by comparing the properties of degradation in the monoculture with those of the proteolytic microbe in

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doi:10.1016/j.jbiosc.2011.07.003

co-culture with hydrogenotrophic methanogens. Three kinds of proteinaceous materials, namely, casein, gelatin, and bovine serum albumin (BSA), were used in the experiments as the representative substrate. *Methanothermobacter thermautotrophicus* is used as hydrogenotrophic methanogen because this related methanogen was often observed in the methanogenic reactors (12,13).

MATERIALS AND METHODS

Organisms *C. proteolyticus* strain CT-1 was isolated from a methanogenic packed-bed reactor degrading artificial garbage slurry under thermophilic condition (55°C). The characteristics of the packed-bed reactor, which includes the supporting material within it, were described previously (12). *M. thermautotrophicus* (JCM1004) was obtained from the Japan Collection of Microorganisms (JCM, Riken, Japan).

Media and growth conditions Basal medium contained 0.8 g of KH_2PO_4, 1.6 g of K_2HPO_4, 1.0 g of NH_4Cl, 2.0 g of $NaHCO_3$, 1.0 g of yeast extract (Wako, Osaka, Japan), 0.1 g of $MgCl_2 \cdot 6H_2O$, 0.2 g of $CaCl_2 \cdot 2H_2O$, 0.8 g of NaCl, 0.1 mg of resazurin, 10 mL of DSMZ medium 131 trace element solution (Deutsche Sammlumg von Mikroorganismen and Zellkulturen, Braunschweig, Germany), 10 mL of DSMZ medium 141 vitamin solution, and distilled water to a final volume of 1 L. Cysteine-HCl·H_2O and $Na_2S \cdot 9H_2O$ were added as reducing agent (1.0 $g \cdot L^{-1}$) to all the culture media. Casein, gelatin, and BSA media were the same as basal medium except that they were supplemented with casein (3.0 $g \cdot L^{-1}$) (Sigma, Tokyo, Japan), gelatin (10.0 $g \cdot L^{-1}$), and BSA (10.0 $g \cdot L^{-1}$), respectively. A gelatin gellan gum plate was the same as basal medium except that it was supplemented with gelatin (3.0 $g \cdot L^{-1}$) and gellan gum (10.0 $g \cdot L^{-1}$), and yeast extract was removed. In addition, basal medium was prepared. Casein medium including supporting material was prepared by adding carbon fiber textiles (CFTs) (20 mm × 100 mm × 2.4 mm), which were pre-autoclaved in distilled water to remove microbial contamination.

Isolation Isolation of proteolytic bacteria was conducted at 55°C in 120-mL serum vials containing 60 mL of casein medium (pH 7.2), which were flushed with O_2-free 80% N_2/20% CO_2 and subsequently autoclaved. The gas phase consisted of 80% N_2/20% CO_2. The seed sludge was inoculated into the casein medium. Positive cultures, judged by ammonium production, were transferred to gelatin gellan gum plates, which were incubated at 55°C under anaerobic conditions with AnaeroPack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). After 10 days of incubation, a colony was picked and re-isolated twice on the same plate. The purity of the colony was checked microscopically, and by sequencing the DNA extracted from the casein medium using the universal primer.

The morphology of the pure culture of strain CT-1 was observed using the microscope (BX50F4; Olympus, Tokyo, Japan). Gram's nature was determined by conventional staining. The DNA from strain CT-1 was obtained as described previously (13). PCR amplification of 16S rRNA genes was done using primers 27f and 1492r (14). The internal 16S rRNA primer Bac806R was also used for full-length sequencing (15). Sequencing of the PCR products was done with a 3130xl Genetic Analyzer (Applied Biosystems, CA, USA) using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The nucleotide sequences were compared with the sequences in the GenBank/EMBL/DDBJ nucleotide sequence databases using the BLAST program.

Comparison between the monoculture and co-culture For the growth experiment (comparison between monoculture and co-culture), 7.1×10^5 cells/mL of *Coprothermobacter* sp. strain CT-1 with and without 2.4×10^5 cells/mL of *M. thermautotrophicus* were added (isolated strain CT-1:*M. thermautotrophicus* = 3:1) into vials (120 mL) containing medium (60 mL, pH = 7.2). The control vials with no addition of cells were similarly incubated. Each of the above 3 types of cultures (co-culture, monoculture, and control) was prepared in triplicate. The growth experiments using casein, gelatin, and BSA media lasted for 7 days, and were performed at 55°C under static conditions. Only the growth experiment using casein medium including CFTs lasted for 10 days and was performed at 55°C under shaker conditions (55 rpm). In addition, the growth experiment using basal medium was conducted in triplicate for 7 days at 55°C under static conditions.

Analytical methods To evaluate the digestibility of the insoluble fraction of casein, dry weights (105°C, 2 h) of suspensions after filtration through glass fiber (0.45-μm) were measured as suspended solid (SS) and compared with those in the control. At the same time, the soluble fraction of casein, which was included in the filtrate, was analyzed by the Bradford assay (16) to measure protein content. Gelatin concentration was analyzed by the Sircol Collagen Assay (Biocolor, UK). BSA concentration was analyzed by the Bradford assay as described above. The volume of gas produced was measured using the water displacement method at a constant room temperature of 25°C. The methane, carbon dioxide, and hydrogen contents of the gas produced were measured using a gas chromatograph equipped with a thermal conductivity detector (GC390B; GL Sciences, Tokyo, Japan) and a stainless-steel column packed with Active Carbon (30/60 mesh; GL Sciences). VFAs were measured by a high-pressure liquid chromatography (HPLC) equipped with an organic acid analysis system (TSK-GEL OApak-A, P; Tosoh, Tokyo, Japan). Total ammonia concentration was measured colorimetrically, as described previously (17). Soluble total organic carbon (S-TOC) was analyzed by a TOC analyzer (TNC-6000; Toray, Tokyo). Total cell number was counted after sampling using a microscope (magnification: ×300) (Nikon, Tokyo). The ratios of *M. thermautotrophicus* to total cells were determined from the ratio of autofluorescence from coenzyme F_{420} to 4′,6-diamino-2-phenylindole (DAPI)-stained cells, using a fluorescence microscope model BX60F-3 (Olympus, Tokyo, Japan). Cell numbers of strain CT-1 and *M. thermautotrophicus* were decided by multiplying each ratio by the total cell number. Carbon content of each substrate was determined by a CHNS/O analyzer (2400II; Perkin-Elmer, Tokyo) or a TOC analyzer.

Nucleotide sequence accession number The nucleotide sequence reported in this paper has been deposited in the DDBJ/EMBL/GenBank databases under accession number AB630185.

RESULTS AND DISCUSSION

Isolation of *Coprothermobacter* strain CT-1 The bacterial strain was isolated from a thermophilic packed-bed reactor. This isolate, strain CT-1, is a thermophilic bacterium and is closely related to *C. proteolyticus* (accession number: CP001145; 99.4% sequence identity), which is reported to utilize protein as a substrate (10,11), and belongs to the order *Thermoanaerobacteriales*. In addition, strain CT-1 was also closely related to an uncultured clone CFB-17 (accession number: AB274506; 97.8% sequence identity) detected in a thermophilic packed-bed reactor degrading garbage slurry (12) and the uncultured clone MUNA04 (accession number: AB114340; 98.8% sequence identity) detected in a thermophilic reactor degrading municipal solid wastes (18). These results show that strain CT-1-related microorganisms were often observed in thermophilic methanogenic reactors degrading organic solid wastes. Strain CT-1 was straight rod, sometimes slightly curved and gram negative in nature, similar to *C. proteolyticus*(11).

Comparison of casein degradation between monoculture and co-culture The co-culture of strain CT-1 with *M. thermautotrophicus* was compared with the monoculture of strain CT-1 using casein as the proteinaceous substrate. Time course of the cell numbers of strain CT-1 and *M. thermautotrophicus* are shown in Fig. 1. Based on the results of microscopic observation, the cell number of strain CT-1 in the presence of *M. thermautotrophicus* increased faster, and reached about 4 times those without *M. thermautotrophicus* after 7 days. The cell number of *M. thermautotrophicus* was only 6.0 ± 2.3% (mean ± standard deviation) of those of the total cells, although initially, it was 25%. These results showed that *M. thermautotrophicus* had a large influence on the growth of strain CT-1, although the percentage of the methanogen in the culture was low. A larger decrease in the insoluble protein content (SS) was observed in the co-culture than in the monoculture (Table 1). In addition, the decrease in the soluble protein content, as measured by Bradford method, was larger in the co-culture than in the monoculture (monoculture: 683 ± 21 mg-BSA/L, co-culture: 292 ± 70 mg-BSA/L, control: 848 ± 8 mg-BSA/L; BSA is used as the standard). At the same time, the concentrations of total

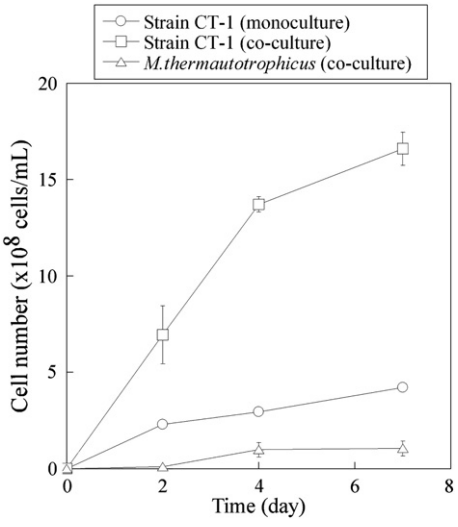

FIG. 1. Time courses of cell numbers in the monoculture (*Coprothermobacter proteolyticus* strain CT-1) and co-culture (*Coprothermobacter proteolyticus* strain CT-1 + *M. thermautotrophicus*) using casein as the protein substrate.