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ORIGINAL PAPER

Isolation and characterization of a new cellulosome-producing *Clostridium thermocellum* strain

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Abstract The anaerobic thermophilic bacterium, *Clostridium thermocellum*, is a potent cellulolytic microorganism that produces large extracellular multienzyme complexes called cellulosomes. To isolate *C. thermocellum* organisms that possess effective cellulose-degrading ability, new thermophilic cellulolytic strains were screened from more than 800 samples obtained mainly from agriculture residues in Thailand using microcrystalline cellulose as a carbon source. A new strain, *C. thermocellum* S14, having high cellulose-degrading ability was isolated from bagasse paper sludge. Cellulosomes prepared from S14 demonstrated faster degradation of microcrystalline cellulose, and 3.4- and 5.6-fold greater Avicelase activity than those from *C. thermocellum* ATCC27405 and JW20 (ATCC31449), respectively. Scanning electron microscopic analysis showed that S14 had unique cell surface features with few protuberances in contrast to the type strains. In addition, the cellulosome of S14 was resistant to inhibition by cellobiose that is a major end product of cellulose hydrolysis. Saccharification tests conducted using rice straw soaked with sodium hydroxide indicated the cellulosome of S14 released approximately 1.5-fold more total sugars compared to that of ATCC27405. This newly isolated S14 strain has the potential as an enzyme resource for effective lignocellulose degradation.

Keywords *Clostridium thermocellum* · Cellulosome · Hyper cellulolytic activity · Rice straw · Degradation

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Introduction

Lignocellulosic plant biomass contains a complex mixture of polysaccharides, such as cellulose, hemicellulose, pectin, and can be utilized as a renewable resource. In particular, cellulose and hemicellulose comprise 40−60% of plant cell walls (McCann and Carpita 2008); efficient conversion of this material by enzymes and/or microorganisms is a desirable goal. However, the plant cell wall is difficult to hydrolyze

because cellulose is surrounded by lignin that has covalent associations with hemicellulose, and cellulose has a tightly packed crystalline structure (Sun and Cheng 2002). Thus, the rate-limiting step in biomass degradation is the conversion of cellulose and hemicellulose polymers to sugars.

Many microorganisms that produce enzymes capable of degrading cellulose and hemicellulose have been reported and characterized (Lynd et al. 2002). Two enzyme systems are known for the degradation of lignocellulose by microorganisms. In many aerobic fungi and bacteria, endoglucanase, exoglucanase, and ancillary enzymes are secreted individually and can act synergistically on lignocellulose. The best studied enzymes are the glycosyl hydrolases of *Trichoderma reesei* (Dashtban et al. 2009). In addition, several anaerobic microorganisms have evolved distinct enzyme systems that involve the formation of large, extracellular enzyme complexes called cellulosomes.

Among the cellulosome-producing microorganisms, *Clostridium thermocellum*, an anaerobic, thermophilic, and spore-forming bacterium, has the most potent cellulose-degrading ability, and thus, attracted wide attention for the source of enzymes to hydrolyze lignocelluloses into glucose and other sugars which then can be converted to useful substances such as ethanol. The cellulosome (2−3.5 MDa) of *C. thermocellum* consists of a large (197 kDa), non-catalytic, multimodular scaffoldingg protein named CipA that includes nine cohesins, four hydrophilic modules, and a family III cellulose-binding module (CBM). The catalytic units are non-covalently attached to scaffolding via high-affinity type I interactions between dockerin domains of the catalytic units with cohesins on the scaffolding (Bayer et al. 2008). The scaffolding with bound subunits is anchored to the cell wall via high-affinity type II interactions between dockerin domains and the cohesins of the anchoring proteins (Fujino et al. 1993). Recent genome sequencing efforts have identified more than 70 dockerin-containing proteins in the genome of *C. thermocellum* ATCC27405 (Demain et al. 2005). Therefore, the cellulosomes of *C. thermocellum* provide a large variety of enzymes and useful enzymatic properties for degradation of complex plant biomass. The structures and properties of cellulosomes from several *C. thermocellum* strains, such as ATCC27405 (DSM1237) (Brown et al. 2007), JW20 (ATCC31449) (Freier et al. 1988), LQR1 (ATCC35609) (Ng et al. 1977), and YS (Lamed et al. 1983), have been characterized (Béguin et al. 1992; Schwarz 2001; Ohmiya et al. 2003; Demain et al. 2005; Bayer et al. 2008). Many of these strains were isolated originally by Viljoen et al. from manure, soil, hay, and compost samples obtained within a confined geographical area in the United States (Viljoen et al. 1926). In contrast, broad screening of a variety of environments for thermophilic anaerobic cellulolytic microorganisms that produce cellulosomes is a useful strategy for identifying new enzymes and finding cellulosomes with efficient lignocellulose degradation ability. In particular, tropical areas such as Southeast Asia have an abundance of agricultural resources, where quick turnover of lignocellulosic biomass occurs compared to other areas. Therefore, screening for cellulolytic microorganisms in tropical areas is promising for isolating new *C. thermocellum* strains capable of producing cellulosomes hyperactive toward lignocellulose.

This report describes the isolation and characterization of *C. thermocellum* S14 with high cellulolytic ability isolated from bagasse paper sludge in Thailand. The cellulosomal enzyme prepared from S14 possessed greater specific activity for microcrystalline cellulose degradation than that of *C. thermocellum* ATCC27405, JW20, and LQR1 strains, but also demonstrated greater saccharification of alkaline-pretreated rice straw. This *C. thermocellum* strain is a useful enzyme resource for lignocellulosic plant biomass degradation.

Materials and methods

Organisms, media, and growth conditions

Clostridium. thermocellum ATCC27405, JW20, and LQR1 were obtained from American Type Culture Collection (ATCC) and Collection of Microorganisms and Cell Cultures (DSMZ). New isolated strain S14 was deposited with the National Institute of Technology and Evaluation Patent Microorganisms Depository (NPMD; Chiba, Japan) as NITE P-627. The *C. thermocellum* strains were grown on BM7 or BM7CO media, pH7.0, containing 1.5 g/l KH_2PO_4 (Wako Pure Chemical, Kyoto, Japan), 2.9 g/l K_2HPO_4 (Wako Pure Chemical), 2.1 g/l urea (Wako

Pure Chemical), 6.0 g/l yeast extract (Difco, Franklin Lakes, NJ, USA), 0.5 g/l cystein hydrochloride, 0.5 mg/l $MgCl_2 \cdot 6H_2O$ (Wako Pure Chemical), 0.0075 mg/l $CaCl_2 \cdot 2H_2O$ (Wako Pure Chemical), and 0.5 mg/l resazurin. BM7CO medium was BM7 medium with 4 g/l Na_2CO_3 (Wako Pure Chemical) added. These media were supplemented with microcrystalline cellulose powder (Sigmacell type-20; Sigma-Aldrich, St. Louis, MO, USA) or cellobiose (Sigma-Aldrich) as carbon sources. BM7 and BM7CO media were degassed by boiling water, followed by bubbling of high-purity nitrogen gas and carbon dioxide, respectively, prior to being anaerobically distributed to Hungate tubes (Bellco Glass, Inc., Vineland, NJ, USA) and/or serum bottles, and subsequently sterilized (20 min, 121°C). *Escherichia coli* DH5α (TaKaRa, Ohtsu, Japan) and plasmids pTAC-1 (TA PCR cloning kit; DynaExpress, Tokyo, Japan) served as the cloning host and vectors, respectively. *Escherichia coli* cells were grown at 37°C on Luria–Bertani medium containing ampicillin (100 μg/ml; Sigma-Aldrich).

Isolation of cellulolytic strains

More than 800 samples were collected from soil, agriculture residues and wastes (e.g., mixture of rice straw, husks, and cotton used for mushroom cultivation, bagasse residues from a sugar cane factory, compost of pineapple peels, sludge from paper, starch and fruit processing factories) in all over Thailand. Approximately 1 g of each sample collected was inoculated directly onto BM7 medium containing 0.5% microcrystalline cellulose powder. Cultures were grown at 60°C for two to three days under nitrogen gas in sealed glass tubes. The cultures were transferred five times to insure they could utilize microcrystalline cellulose as the sole carbon source and were not living solely on nutrients in the environmental sample. Thereafter, each culture was maintained in broth tubes as mixed cultures and plated out on anaerobic Hungate roll tubes (Hungate 1969) containing 1.0% microcrystalline cellulose and 1.5% agar (Wako Pure Chemical). Individual colonies that produced a clear zone due to degradation of cellulose were collected from the roll tubes and inoculated in BM7 medium containing microcrystalline cellulose. Then, individual cultures were plated out on roll tubes containing 0.5% cellobiose. Formed colonies were collected and tested for cellulose degradation in BM7 medium with microcrystalline cellulose. The pure cultures of cellulose degraders were obtained after five-time repeated single colony isolation procedures on cellobiose medium.

Identification of new hypercellulolytic strain

To select new thermophilic anaerobic microorganisms possessing hypercellulolytic activity, pure isolated strains from the environmental samples were compared to *C. thermocellum* ATCC27405. Cellulolytic isolates were grown on BM7 medium with 0.5% cellulose powder in anaerobic culture tubes. Samples were taken after three days of growth, and culture supernatants were obtained by centrifugation at $7{,}000 \times g$ for 5 min at 4°C. The supernatants were treated as enzyme solutions for turbidity assays using microcrystalline cellulose powder as described by Johnson et al. (1982). The turbidity assay was performed using 10% (vol/vol) culture supernatant, 0.06% (wt/vol) microcrystalline cellulose (Sigmacell type-20), 0.1 M sodium acetate buffer (pH 6.0), 5 mM $CaCl_2 \cdot 2H_2O$, and 10 mM dithiothreitol (DTT) in Hungate tubes. A reduction in turbidity was measured as a decrease in absorbance at 660 nm. Cellulose degradation ability between isolated strains and *C. thermocellum* ATCC27405 was compared using a decrease in turbidity.

16S rRNA gene cloning, nucleotide sequencing

Preparation of chromosomal and plasmid DNA, and transformations were conducted using standard procedures or according to supplier protocols. The genomic DNA was subjected to PCR with the oligonucleotide primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGCTACCTTGTTACG ACTT-3′). PCR was performed with standard conditions for Ex Taq polymerase (Takara, Kyoto, Japan) according to manufacturer's instructions. The amplified fragments were purified with a gel extraction kit (Qiagen, Hilden, Germany) and subcloned to pTAC-1 followed by transformation into *E. coli* JM109. Plasmids were extracted with the QIAprep Spin Miniprep kit (Qiagen). After checking the sequence heterogeneity by restriction fragment length polymorphism (RFLP) analysis with *Eco*RI, the nucleotide sequence of the 16S rRNA gene clone was determined by the dideoxy

chain termination method. Sequence data were analyzed with the Genetyx ver. 8.0 software package (Genetyx Corporation, Tokyo, Japan). Nucleotide and sequence data were analyzed and compared using BLAST software at NCBI (http://www.ncbi.nlm.nih.gov). The partial 16S rRNA gene sequence of the organism has been deposited in GenBank under accession number HQ315888.

Physiological properties of hypercellulolytic strain S14

Physiological characterization of *C. thermocellum* strains was obtained using standard protocols conventionally used in bacterial systems (Gerhardt et al. 1981). Growth measurement on various carbon sources was determined by cultivating strain S14 for six days at 60°C in BM7 liquid medium supplemented with xylan (birchwood and oat-spelt xylan, Tokyo Chemical Industry, Japan), galactomannan (Megazyme, Ireland), soluble starch (Wako Pure Chemical), Pectin (from Apple; Wako Pure Chemical), cellobiose (Sigma-Aldrich), glucose, sucrose, maltose, xylose, galactose, lactose, mannose, or sorbitol (all from Wako Pure Chemical). Optimum growth conditions were determined by cultivating at 37−75°C in BM7 liquid medium with cellobiose. For organic acid and ethanol production, *C. thermocellum* strains were cultured in BM7CO medium with 1% microcrystalline cellulose. The organic acid content was measured using a high-performance liquid chromatograph (HPLC) (Shimadzu, Kyoto, Japan) equipped with an electric conductivity monitor (CDD-10A; Shimadzu). The samples were separated at 45°C on a Shimpack SPR-H column (Shimadzu) according to the manufacturer's protocol. A gas chromatograph (model GC-2014, Shimazu) with a flame-ionization detector was used to measure ethanol concentration in samples under the following conditions: glass column (8.0 mm × 3.2 m) packed with Chromosorb 103 (60/80 mesh); temperatures of column, injector, and detector, 185, 175, and 250°C, respectively; helium carrier gas flow rate, 20 ml/min; with *n*-propanol as an internal standard.

Preparation of cellulosomal enzymes

Cellulosomes were prepared from cell-free broth from cultures grown in BM7CO supplemented with 1% microcrystalline cellulose using the affinity digestion method (Morag et al. 1992). Cultures were centrifuged and the cell-free broth was incubated with phosphoric-acid-swollen-cellulose (100 mg/l cell-free broth) (Wood and Kellogg 1988) overnight at 4°C for cellulase binding to cellulose. On the following day, amorphous cellulose with bound enzymes was centrifuged and re-suspended in stabilizing buffer [50 mM Tris–HCl (pH 7.0), 50 mM $CaCl_2$, 50 mM EDTA]. The amorphous cellulose-bound enzymes were incubated and dialyzed in dialysis tubes (cellulose ester, Spectra/Por, 1 kDa cut-off) at 60°C against deionized water to enhance amorphous cellulose degradation by the enzymes. Deionized water was changed every 3 h to avoid inhibition of cellulases by the degradation product, cellobiose. The suspension cleared within 6 h, and purified cellulase fraction was obtained after centrifugation of the clarified solution.

Enzyme and protein assays

All assays were performed at 60°C in 0.1 M sodium acetate buffer (pH 6.0) with 5 mM $CaCl_2$ under static conditions for 20 min except for Avicelase and filter paper hydrolysis activity wherein reaction was conducted under shaking at 100 rpm for 30−60 min. Avicelase, endoglucanase, xylanase, mannanase, and pectinase activities were measured by the amount of reducing sugars liberated from Sigmacell type-20, carboxymethylcellulose, oat-spelt xylan, carob galactomannan, and polygalacturonic acid (all from Sigma-Aldrich), respectively, with 0.3% (v/w) final concentration (Wood and Kellogg 1988). Reducing sugars were determined using the Somogyi−Nelson method (Nelson 1944). The β-glucosidase, β-xylosidase, and α-L-arabinofuranosidase activities were based on measurement of the release of *p*-nitrophenol from *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl β-D-xyloside, and *p*-nitrophenyl β-D-arabinopyranoside (all from Sigma-Aldrich), respectively (Wood and Kellogg 1988). One unit of enzymes releases 1 μmol equivalent of glucose, xylose, mannose, galacturonic acid or *p*-nitrophenol per min. Filter paper hydrolysis activity (FPase) was determined by the standard method (Wood and Kellogg 1988) using Whatman No. 1 filter paper, and was expressed in filter paper units (FPU). One FPU was defined as the amount of enzyme capable of producing 1 μmol of

reducing sugars in 1 min. Protein concentrations were determined with the Pierce BCA assay kit (Thermo Fisher Scientific, Rockford, USA) with bovine serum albumin as the standard.

Gel filtration chromatography

The cellulosome preparations were subjected to gel filtration on a XK16/100 column of Sepharcyl S-500 (GE Healthcare) equilibrated with 50 mM Tris–HCl buffer, pH 7.5, containing 150 mM NaCl, and eluted with the same buffer.

Gel electrophoresis and microscopic observation

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 5−20% gradient polyacrylamide gels (ATTO, Tokyo, Japan) following manufacturer's instructions. Samples used for SDS-PAGE were boiled for 5 min in sample buffer that contained DTT. *C. thermocellum* ATCC27405 and isolated S14 cells were observed using a scanning electron microscope (Jeol JSM-6320F, Tokyo, Japan) according to the manufacturer's instructions. The specimen for scanning electron microscopy was prepared by osmium fixation and critical-point drying (Gerhardt et al. 1981).

Estimation of cellular protein for monitoring the cell growth

Monitoring the cell growth in BM7CO supplemented with cellulose was based on cellular protein estimation as described by Bensadoun and Weinstein (1976). A 1 ml aliquot of the culture broth was centrifuged for 10 min at 7,000×*g*. The pellets were washed with the same volume of 50 mM sodium phosphate buffer (pH 7.0) and incubated with 4 ml of sodium deoxycholate (2%) (Wako Pure Chemical) for 20 min. A 1 ml volume of 24% trichloroacetic acid (Wako Pure Chemical) was added to the suspension and centrifuged at 7,000×*g* for 10 min. Under these conditions, the cells burst, and cellular proteins were found in the pellet. The protein concentration was determined by the BCA protein assay kit with bovine serum albumin as the standard and used for monitoring the bacterial growth in the presence of cellulose.

Adherence assay

Measurements of cell adherence to cellulose were carried out by the turbidity assay using microcrystalline cellulose powder as described by Bayer et al. (1983). A washed cell suspension (approximately 1 mg of cells in 1 ml PBS) was added to 4 ml of PBS buffer containing Sigmacell. The suspension was brought to a total volume of 5 ml with 5% microcrystalline cellulose as final concentration. The suspension was vortexed for 1 min, and the cellulose containing the adhered bacterial cells was allowed to settle at room temperature for 60 min. The turbidity (absorbance at 400 nm) of supernatant was measured and compared with control tubes wherein PBS was substituted for the cellulose suspension.

N-terminal amino acid sequencing

Subunit proteins of the cellulosomes were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Millipore Corporate, MA, USA) by MINITrans-Blot electrophoretic transfer cell (Bio-Rad Laboratories, Richmond, USA). The protein bands were stained with 0.1% Ponceau red (Sigma-Aldrich) and cut out and were then sequenced by using a model 477 protein sequencer (Applied Biosystems, Foster City, USA).

Saccharification of rice straw pretreated with sodium hydroxide

Rice straw was purchased from the Miyahara store (Nagano, Japan). It was ground and sieved through a 0.5-mm mesh screen (ZM-100; Retsch, Haan, Germany). A total of 40 g of the milled rice straw was soaked in 400 ml of 1% (wt/vol) NaOH solution at 30°C for 3 days. After pretreatment, the wet solid was washed with deionized water until neutral pH and used for moisture measurement, sugar analysis and saccharification experiments with the cellulosome preparations. Moisture content was measured by drying in an oven at 70°C for 2 days. The total sugar content and sugar composition of the rice straw pretreated with NaOH were analyzed following the NREL Chemical Analysis and Testing Standard Procedure as described below. The oven dried sample was taken to a 72% sulfuric acid hydrolysis at 30°C for 60 min, followed by 3%

sulfuric acid hydrolysis at 121°C for 60 min. The hydrolysate was neutralized to pH 6.0 by calcium carbonate (Wako Pure Chemical) and vacuum filtered through a filtering crucible. Total sugar content in the filtrate was determined by the phenol–sulfuric acid method with glucose as standard (Wood and Kellogg 1988). Monosaccharide's were analyzed by HPLC (Shimadzu) on a Bio-Rad Aminex HPX-87P column (Bio-Rad Laboratories) operated at 80°C with MilliQ water (0.6 ml/min) and a refractive index detector (Shimadzu RID-10A).

Enzymatic saccharification of the pretreated rice straw was performed using the cellulosome preparations containing 2 μg protein per milligram substrate at a substrate loading of 0.5% (wt/vol) by shaking at 100 rpm at 60°C in a 0.1 M sodium acetate buffer (pH 6.0) containing 5 mM $CaCl_2$. A sample was taken periodically and measured for the released total sugars and monosaccharaides as described above.

Results

Isolation of hypercellulolytic *C. thermocellum* strains

More than 800 samples of agricultural residues, wastes and soil were individually cultivated in BM7 medium containing 0.5% microcrystalline cellulose as the sole carbon source. Many environmental samples could degrade and ferment the microcrystalline cellulose. To select microorganisms possessing high cellulose-degrading ability, enrichment culturing was performed. After repeating the enrichment culture several times, single colonies were isolated by the roll tube method using cellulose or cellobiose as a carbon source. Eventually, monoculture candidates were obtained from more than 120 samples with high cellulolytic activity. To choose monoculture candidates possessing high cellulolytic ability, cellulose-grown cultures of each candidate were compared to that of strain *C. thermocellum* ATCC 27405 using a turbidity test. Several monocultures designated N44, OP70, SP18, MP17, and S14 possessed greater and more rapid cellulose degradation activity than that of ATCC27405, which have homology of greater than 99% toward 16S rRNA sequence of ATCC27405 (data not shown). The *C. thermocellum* S14 strain had the greatest and most rapid cellulolytic activity of the monocultures.

To confirm whether the S14 high cellulolytic ability was due to cellulosome formation, each cellulosome enzyme mixture from *C. thermocellum* ATCC27405, JW20, LQR1, and S14 was prepared using affinity digestion from cellulose-grown cultures, and turbidity testing using microcrystalline cellulose was conducted. Enzymes from S14 had the greatest and most rapid cellulose degradation rates compared to other cellulosomal enzymes (Fig. 1). To characterize the cellulosomal enzyme of S14, several glycoside hydrolase activities were compared to that of cellulosomal enzymes from ATCC27405 and JW20 (Table 1). Cellulosomal enzyme of S14 showed higher specific activities than those of ATCC27405 and JW20 for all assayed glycoside hydrolases, except for β-xylosidase and mannanase. Cellulosomal enzyme of S14 indicated 3.4 and 5.6-fold greater hydrolytic activity than that of ATCC27405 for microcrystalline cellulose (Avicelase).

In addition to the high specific activities of the cellulosomes, S14 produced larger amounts of cellulosomes than ATCC27405. S14 produced approximately 240 mg/l cellulosomal proteins in the culture medium, whereas ATCC27405 produced 60−70 mg/l.

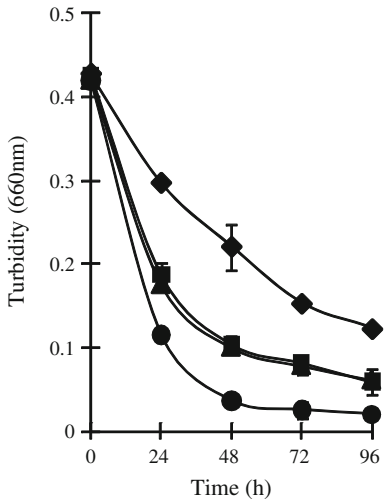

Fig. 1 Solubilization of microcrystalline cellulose by cellulosomes prepared from *C. thermocellum* type strains and S14. Turbidity assay was conducted in duplicate using cellulosomes containing 24 μg protein per milligram cellulose according to the conditions described in Materials and Methods. *Closed circles*, *triangles*, *squares*, and *diamonds* indicate turbidity test values using cellulosomes from S14, ATCC27405, LQR1, and JW20, respectively

Table 1 Comparison of glycoside hydrolase activities of the cellulosomes prepared from *C. thermocellum* ATCC27405, JW20, and isolated S14 strains

Glycoside hydrolases	Enzymatic activities (units/mg proteins)[a]		
	ATCC27405	JW20	S14
Avicelase	0.56 ± 0.26	0.34 ± 0.06	1.93 ± 0.29
FPase[b]	0.037 ± 0.01	0.026 ± 0.01	0.068 ± 0.02
CMCase	1.10 ± 0.12	1.1 ± 0.31	1.50 ± 0.42
β-Glucosidase	<0.001	<0.001	<0.003
Xylanase	1.70 ± 0.24	1.36 ± 0.16	3.60 ± 0.9
β-Xylosidase	<0.001	<0.001	<0.002
α-arabinofuranosidase	<0.001	<0.001	<0.004
Mannanase	0.081 ± 0.06	0.082 ± 0.03	0.083 ± 0.05
Pectinase	0.05 ± 0.01	0.05 ± 0.02	0.16 ± 0.09

[a] Glycoside hydrolases were assayed using the cellulosome preparations containing 20−50 μg protein. Values are means of three determinations ± SD

[b] FPase, filter paper hydrolyzing activity (FPU)

Morphologic and physiologic properties of strain *C. thermocellum* S14

New strain *C. thermocellum* S14 possessed 99% similarity with type strain ATCC27405 in 16S rRNA sequence; however enzymatic activities were substantially distinct from those of strains ATCC27405 and JW20. To confirm whether S14 and ATCC27405 differed in other properties, morphological features (using scanning electron microscopy), growth rates on various carbon sources, and fermentation products from cellulose were compared. Scanning electron micrographic images of ATCC27405 and S14 grown in BM7CO medium supplemented with either microcrystalline cellulose or cellobiose are shown in Fig. 2. S14 exhibited morphological features similar to ATCC27405, such as rod-shaped cells and oval spores. However, the cell surface of S14 was rather smooth compared to the protuberance structures of the cell surface of ATCC27405. Especially, cells grown in cellulose medium had very few protuberances.

The physiological properties of S14 and ATCC27405 are compared in Table 2. S14 could grow at a higher temperature of 70°C and a more alkaline pH of 9.0, under which conditions ATCC27405 and other reported *C. thermocellum* strains can not grow. Unlike ATCC27405, S14 grew quickly on glucose and sorbitol. Although *C. thermocellum* strains commonly produce yellow affinity substance (YAS) that is considered carotenoid-like compound and enhances cellulolytic activity (Ljungdahl et al. 1988), no yellow color was found during degradation of cellulose in the S14 cultures. Ethanol, acetate, lactate and propionate were detected as major fermentation products, as in the case of the reported *C. thermocellum* strains (Ozkan et al. 2001). However, amounts of acetate and propionate produced by S14 were distinctively larger than those by the reported strains.

As S14 did not show clear protuberance structures on cell surface when grown on cellulose, its adherence ability was examined. A measured amount of cellulose was mixed with a washed cell suspension. After settling of the cellulose together with the adhered cells, the residual turbidity was measured. In the presence of cellulose, the turbidity of a cell suspension of ATCC27405 grown on cellulose decreased to approximately 40% of the initial A400, whereas the turbidity of a cell suspension of S14 grown on cellulose decreased to only 70% of the initial A400, indicating less adhering ability which reflects fewer protuberances on cell surface (Fig. 2. EX, ST). Intriguingly, the cellobiose-grown S14 cells showed stronger adherence ability to cellulose than cellulose-grown cells, which accords with the cell surface structure with more protuberances (Fig. 2. CB).

Characterization of the cellulosomes prepared from strain S14

The cellulosome preparations from S14 and ATCC27405 were subjected to gel filtration on Sephacryl S-500. The cellulosomal enzymes eluted

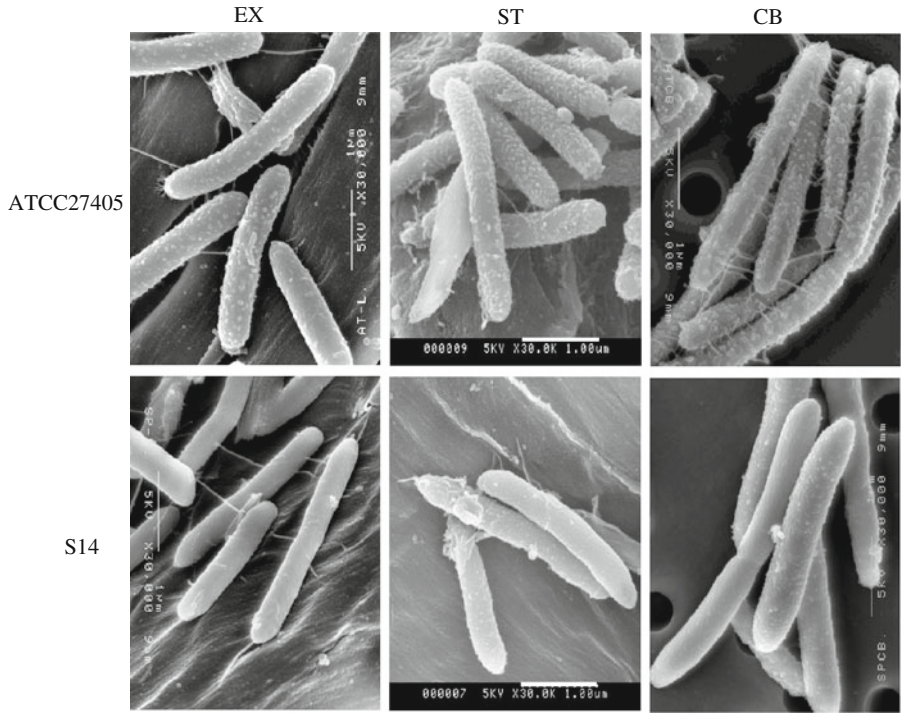

Fig. 2 Scanning electron micrographs (SEMs) of *C. thermocellum* ATCC27405 and S14. Both strains were grown in BM7CO medium containing 0.5% microcrystalline cellulose or 0.5% cellobiose at 60°C, respectively. EX and ST indicate SEMs for each strain at exponential (approximately 18 h after inoculum) and stationary (approximately 48 h after inoculum) growth phases, respectively, in cellulose medium. CB shows SEMs of both strains from cellobiose grown cultures at exponential growth phase (approximately 12 h after inoculum). *White bars* indicate scales of 1 μm

as a large peak having Avicelase activity accompanied with several small peaks. Although elution of the cellulosomes were retarded presumably due to interaction between the cellulsomes and the gel material (Mori 1992), which is common phenomenon with glycoproteins such as cellulosomes, cellulosomes of S14 appeared to be larger than those of ATCC27405 from the eluting positions.

To elucidate the origin of the high cellulolytic ability of S14, the SDS-PAGE pattern of cellulosomal enzymes of S14 was compared to that of ATCC27405. The cellulosomal enzymes of S14 had a partly different protein pattern and ratio than that of ATCC27405 (Fig. 3). To identify the major distinct protein bands indicating cellulosomal enzymes of S14, the N-terminal amino acids of the 200, 75 and 60 kDa subunits were sequenced and identified as the scaffolding protein CipA, Cel48S, and Cthe0821, respectively. The difference in molecular mass between CipA from ATCC27405 and from S14 is presumably due to the degree of glycosylation. The Cel48S and cellulosomal subunit band corresponding to 95 kDa were detected as major subunits in both strains but at higher relative concentrations in S14 cellulosome. In contrast to these proteins, Cthe0821, which is an endoglucanase belonging to glycoside hydrolase family 5 (Carbohydrate-Active EnZymes Database, http://afmb.cnrs-mrs.fr/pedro/CAZY), was abundant in the cellulosomes of ATCC27405 (Raman et al. 2009), but scarce in the cellulosomes from S14 based upon N-terminal sequencing and SDS-PAGE pattern.

The cellulolytic ability of cellulosomes is strongly inhibited by cellobiose, which is a major end product of cellulose hydrolysis (Johnson et al. 1982). To measure sensitivity to cellobiose of the S14 cellulosomal enzyme, cellulolytic activity was measured with microcrystalline cellulose in the presence of different cellobiose concentrations (1 and 5 mM) (Fig. 4). When cellulosomes of ATCC27405 and S14 were incubated with microcrystalline cellulose in the presence of 1 mM cellobiose, slight inhibition of activity was observed for ATCC27405. However,

Table 2 General properties and fermentation products of *C. thermocellum* ATCC27405 and S14

General properties and fermentation products	*C. thermocellum* strains	
	ATCC27405	S14
Cell shape	Rod	Rod
Cell length (μm)[a]	3.0−4.0	2.8−4.0
Spore forming	+	+
Maximum growth temperature (°C)	65	70
Growth pH range	6.0−7.5	6.0−9.0
Growth on various carbon sources		
Xylan (birchwood and oat spelt)	−	−
Xylose	−	−
Cellobiose	+	+
Glucose	±[b]	+
Fructose	+	+
Sorbitol	−	+
Fermentation products from 1% cellulose		
Pigment productions	Yellow	Cream
Cellulose hydrolyzed (%)[c]	98.8%	99.2%
Ethanol (g/l)[d]	1.09 ± 0.01	1.90 ± 0.09
Acetate (g/l)[d]	1.49 ± 0.02	3.72 ± 0.18
Lactate (g/l)[d]	2.43 ± 0.06	0.74 ± 0.11
Propionate (g/l)[d]	0.25 ± 0.01	1.23 ± 0.01

[a] Cell length was measured from picture of SEM

[b] The growth was very weak and took 4 days to be recognized

[c] Cultures that contained microcrystalline cellulose were centrifuged at 7,000×*g* for 5 min. After two times of washing with 50 mM sodium acetate buffer (pH 6.0), pellets were dried at 70°C for 3 days. Percentage of hydrolyzed cellulose was calculated by measurement of weight of dried pellets

[d] Average from duplicate fermentations on 1% (wt/vol) microcrystalline cellulose

almost no inhibition occurred for those of S14. When the cellobiose concentration was increased to 5 mM, the cellulolytic ability of the ATCC27405 cellulosomes was strongly inhibited, but significant level of the activity of the S14 cellulosomes was retained (Fig. 4). These results clearly indicate that the cellulosomes of S14 possess greater resistance to cellobiose inhibition than those of ATCC27405.

Saccharification of alkaline-pretreated rice straw by cellulosomes from S14 and ATCC27405

Polysaccharide structures of lignocellulosic biomass, such as rice straw and corn stover, are more complex than those of pure microcrystalline cellulose due to networks of lignin, cellulose, and hemicellulose. Therefore, to test whether the cellulosome from S14 possesses the ability to degrade natural lignocellulose, saccharification tests were conducted using alkaline-pretreated rice straw. To eliminate a decrease in enzyme accessibility by lignin, rice straw was pretreated with 1% NaOH at 30°C for 3 days. The total amount of sugars from this alkaline-pretreated rice straw was determined to be approximately 642 mg/g by phenol–sulfuric acid method. The monosaccharide composition of the pretreated rice straw was analyzed in triplicate by HPLC using its sulfuric acid hydrolyzates and determined as follows in the weight percent of the dry matter: glucose 50.6 ± 0.1%, xylose 28.6 ± 0.1%, arabinose 4.7 ± 0.1%. When pretreated rice straw was hydrolyzed by the cellulosomes from S14 and ATCC27405, 1.31 and 0.89 mg/ml sugars were released from 0.5% (wt/vol) solid substrates, which correspond to saccharification rates of 40.8 and 27.7%, respectively (Fig. 5). Cellobiose and xylobiose were major products whereas glucose and xylose were only minor sugar components in both hydrolyzates (data not shown). Cellobiose and xylobiose levels of approximately 70.5−77.3% and 13.7−20.7%, respectively, were found for released total sugar.

Discussion

Several studies have reported isolation and characterization of anaerobic cellulolytic microorganisms from agricultural wastes in tropical areas. Sukhumavasi et al. isolated and characterized the anaerobic

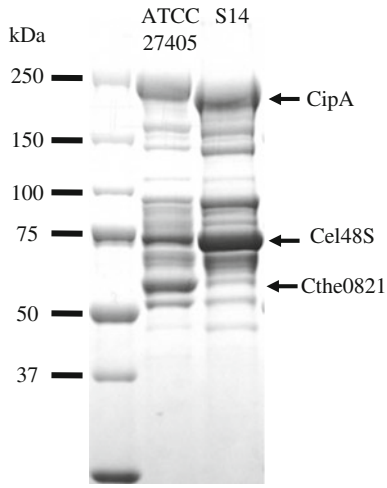

Fig. 3 SDS-PAGE pattern of cellulosomal enzymes prepared from *C. thermocellum* ATCC27405 and S14. Samples containing 20 μg potein were boiled for 10 min in sample buffer prior to SDS-PAGE on 4−20% gradient polyacrylamide gels. Proteins in gels were detected by Coomassie R-250 staining. *Arrows* indicate cellulosomal subunits identified by N-terminal amino acids sequence analysis

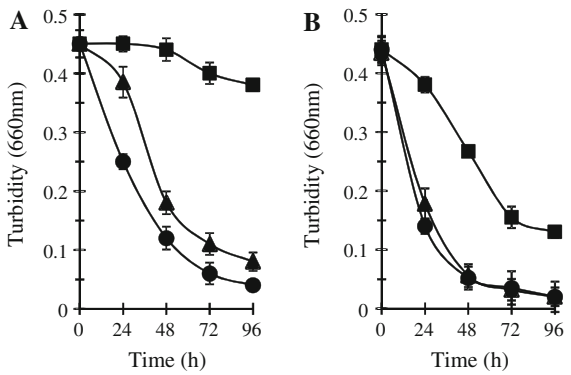

Fig. 4 Effect of cellobiose inhibition on cellulosomes prepared from **A** *C. thermocellum* ATCC27405 and **B** S14. Cellulosomes prepared from each strain were subjected to turbidity assay with microcrystalline cellulose. Turbidity assay was conducted in duplicate using cellulosomes containing 24 μg protein per milligram cellulose in the presence of 0 (*closed circle*), 1 (*closed triangle*), and 5 mM (*closed square)* cellobiose

and cellulolytic bacterium *C. josui*, which produces a cellulosome, from Thai composts (Sukhumavasi et al. 1988). Virunanon et al. also reported isolation of solventogenic-cellulolytic clostridia through a screening process using cellulolytic activity and butanol tolerance in selective media from decomposed sources, cow feces, and dry grass in Thailand (Virunanon et al. 2008). These isolated cellulolytic *Clostridium* species belong to mesophilic clostridia active below 50°C and possess very weak cellulose degrading activity. This is the first report for isolation and characterization of thermophilic, anaerobic, and cellulolytic bacteria from tropical areas. Potent cellulolytic bacterial strains were obtained and the representative strain S14 was identified as *C. thermocellum.* The newly isolated S14 strain can degrade cellulose more rapidly than *C. thermocellum* type strains and produces cellulosomes with greater cellulolytic and xylanolytic activities than those from other strains.

Intriguingly, S14 and other isolates had very few protuberance structures on the surface of cells grown on cellulose despite that they had strong cellulolytic activity. Meanwhile, the cellobiose-grown S14 cells had considerable amounts, although less than ATCC27405, of protuberances on the cell surface (Fig. 2 CB). Bayer et al. (1986) reported that after attachment to the insoluble cellulose the protuberance protracted rapidly to form fibrous 'contact corridors', which indicated detachment of cellulosomes from cell surface occurred in the presence of cellulose. The above-mentioned observations and information

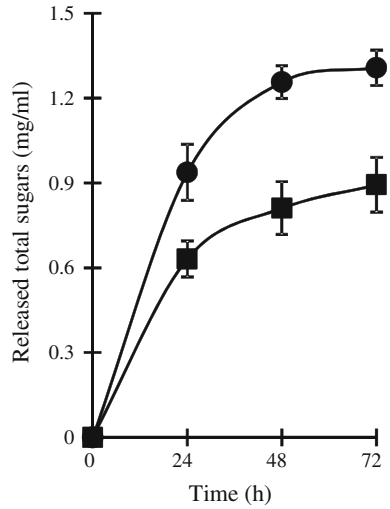

Fig. 5 Saccharification of alkaline-pretreated rice straw using cellulosomes prepared from strains ATCC27405 and S14. *Closed squares* and *circles* indicate saccharification profiles using cellulosomal enzymes from ATCC27405 and S14, respectively. Saccharification testes were carried out in duplicate using cellulosomes containing 2 μg protein per milligram rice straw according to the conditions described in "Materials and methods" section

suggest that S14 is more efficient in releasing cellulosomes, especially, in the medium containing cellulose. Actually, S14 accumulated in the medium nearly four times larger amounts of cellulosomes than ATCC27405.

Cellulosomes of S14 seemed to be larger than those of ATCC27405. The eluting position of the S14 cellulosomes on gel filtration was similar to that of cellulosomes from strain YM4 (Mori 1992) which had a molecular mass of approx. 3.5 MDa (Mayer et al. 1987), while the ATCC 27405 cellulosomes eluted at a similar position of the JW 20 (ATCC31449) cellulosomes with 2.0−2.5 MDa (Mori 1992; Mayer 1987). These comparisons suggest that the S14 cellulosomes, being estimated roughly at 3.5 MDa, are much larger than those of ATCC type strains.

The strain S14 and its cellulosomes have properties advantageous in practical applications; it has a wide temperature and pH growth range and produces large amounts of cellulosomal enzymes in the medium. Its cellulosomes are more active toward both microcrystalline cellulose and lignocellulose, such as rice straw, with higher resistance to cellobiose inhibition, compared to the cellulosomes of the ATCC type strain. Therefore, S14 and its cellulosomes can be considered the promising tools for utilization of cellulosic resources.

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