Characterization of New Strains of *Clostridium thermocellum* and the *celA* Gene from a Strain

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Two strains of thermophilic cellulolytic anaerobes, Nos. 138 and 183, were isolated from soil and compost, respectively, and identified as *Clostridium thermocellum*, based on their morphological, physiological and genetic characteristics. These two isolates decomposed cellulose more efficiently than the type strain, *C. thermocellum* ATCC 27405. The *celA* gene of strain No. 138 was cloned onto vector plasmid pBR322 and the hybrid plasmids obtained were introduced into *Escherichia coli* cells. The cleavage map of the cloned *celA* gene and the extent of CM-cellulase expression of the cloned gene in *E. coli* were the same as those of the *celA* gene from ATCC 27405.

The cellulosic biomass, which is abundant and renewable, is an important resource for the production of useful chemicals, such as alcohol and various organic acids. Thus, microbial cells with high cellulosic biomass-degrading activity have been extensively screened for. Cellulase produced by *Trichoderma reesei* and similar fungi is a typical enzyme much studied for possible practical application to the production of ethanol. In addition to fungal microbes with high cellulase-producing activity, many cellulolytic bacteria have also been isolated. *Clostridium thermocellum* cells secrete cellulase and convert cellulose directly to ethanol at high temperatures. Thermostable cellulases produced by thermophiles such as *C. thermocellum* are of particular interest, and many reports on the isolation of thermophilic cellulolytic anaerobes and their cellulases,^{1~6)} and cellulase genes^{7~9)} have been published.

Recently, we isolated two strains of thermophilic cellulolytic clostridia for use for the direct production of ethanol from the cellulosic biomass. In this paper, we describe the isolation and characterization of these cellulolytic

thermophiles that grow under anaerobic conditions. The cloning of the *celA* gene from one isolate and its expression in *E. coli* cells were also studied.

Materials and Methods

Strains. The strains used were *C. thermocellum* Nos. 138 and 183, isolated from soil and compost, respectively; *C. thermocellum* ATCC 27405 and ATCC 31549 were used for reference. *Escherichia coli* strains JM83, C600 and HB101 were used as host cells for the transformation of the *celA* gene. Plasmid pCT 104 containing the *celA* gene of *C. thermocellum* was a gift from Prof. Aubert (Institut Pasteur, France).

Culture conditions. Thermophilic anaerobic cells were grown at 60°C under strict anaerobic conditions (N₂ gas exchange) in GS medium¹⁰⁾ containing cellulose as the sole source of carbon. For the cellulolytic clostridia, DSM medium¹¹⁾ and K medium¹²⁾ modified with DSM medium were used, which contained cellulose or cellobiose. *E. coli* cells were grown in L-broth. For the selection and cultivation of *E. coli* transformants, ampicillin (50 µg/ml) was added to the L-broth.

Analyses. The ethanol, acetic acid and lactic acid produced in the broth were assayed by gas chromatography.

Glucose was measured enzymatically with a Glucostat (Fujisawa Pharmaceutical Co., Japan). The reducing sugar concentration was measured with 3,5-dinitrosalicylic acid according to the method described by Miller.¹³⁾

Measurement of the GC content. Whole-cell DNA of the clostridia was extracted by the alkaline extraction procedure of Birnboim and Doly.¹⁴⁾ The guanosine + cytosine (GC) content of the DNA was measured with a DNA-GC kit (Yamasa Shoyu Co., Ltd., Japan). The DNA decomposed by nuclease P₁ was measured as dAMP, dGMP, dCMP and dTMP by HPLC on a Lichrosorb RP-18 column.

DNA homology. Whole-cell DNA of *C. thermocellum* ATCC 27405 extracted by the above procedure was nicked with DNase I and some of the nucleotides were replaced with radioactive nucleotides, [³H]dTTPs. Nick-translated DNA of strain ATCC 27405 was hybridized with the DNA of strains Nos. 138 and 183, and ATCC 27405, which was fixed on a membrane filter. The radioactivity retained on the filters was measured with a scintillation counter. The amount of radioactivity in the homologous filters was taken as 100% homology and compared with the activity of the heterologous filters.

Enzyme assay. Clostridial cells were grown to the late-log phase in K medium containing 1% cellulose at 60°C. The culture filtrate was used for assaying CM-cellulase and avicelase activities. The collected cells were suspended in 0.1 M potassium phosphate buffer (pH 7.0) and then sonicated at 90 kHz for 30 min at 0°C in a Kubota Model 200M Insonator. The sonicates were centrifuged and the clear supernatants were used for assaying β -glucosidase activity. *E. coli* cells carrying plasmid DNA were grown overnight in L-broth containing 50 μ g/ml ampicillin. The cells were collected by centrifugation, suspended in PC buffer (50 mM K₂HPO₄ and 12.5 mM citric acid, pH 6.3) and then sonicated at 90 kHz for 10 min as described by Cornet *et al.*⁷⁾

CM-cellulase activity was assayed by measurement of the reducing sugars produced. One milliliter of the enzyme solution was added to 1 ml of 1% CM-cellulose (CMC) dissolved in 0.1 M potassium phosphate buffer (pH 7.0), followed by incubation at 60°C for 1 hr. After the mixture had been boiled for 10 min, its reducing sugar concentration was measured by the method described above. For assaying CM-cellulase activity in the crude extract of *E. coli*, 1 ml of a 1.5% CMC solution in PC buffer was mixed with 50 μ l of the enzyme solution, and then the mixture was incubated at 60°C for 1 hr. The reducing sugars formed were measured by the Somogyi-Nelson method.¹⁵⁾

Avicelase activity was also assayed by measurement of reducing sugars. One milliliter of the enzyme solution was mixed with 1 ml of 1% Avicel in PC buffer, followed by incubation at 60°C for 3 hr.

β -Glucosidase activity was assayed by measurement of

the glucose formed. One milliliter of 2% cellobiose in PC buffer and 50 μ l of the enzyme solution were mixed, and then the mixture was incubated at 60°C for 30 min. After the reaction mixture had been boiled for 10 min, its glucose concentration was measured with a Glucostat.

Proteins in the culture filtrates and crude extracts of clostridial and *E. coli* cells were determined by the methods of Bradford¹⁶⁾ and Lowry *et al.*,¹⁷⁾ respectively.

Cloning of the *celA* gene. Based on the reported base sequence⁹⁾ of the *celA* gene from *C. thermocellum* ATCC 27405, two kinds of probe DNAs (each 30 merlons) were synthesized and their 5' ends were labeled with ³²P. The *celA* gene of strain No. 138 was detected as hybridization of the chromosomal DNA, which was digested with restriction endonuclease *Hind*III, with the two probe DNAs. The *Hind*III fragment containing the *celA* gene was removed from the agarose gel and inserted into the same restriction site of plasmid pUC19.

Chemicals. [³H]dTTP and [γ -³²P]ATP were purchased from Amersham Corp., England. Cellulose (KC-Floc) was purchased from Sanyo Kokusaku Pulp Corp., Japan. Restriction endonucleases and T₄ DNA ligase were obtained from Takara Shuzo Co., Ltd., Japan.

Results

Isolation of cellulose-fermenting thermophilic anaerobes

Approximately 770 soil and compost samples were individually incubated at 60°C in GS medium (10 ml of medium/tube) containing 1% cellulose as the sole source of carbon. A small portion of well-grown cultures was transferred to new medium for enrichment of microbial cells with cellulose-utilizing activity. From two well-grown cultures, single colonies were formed in the agar medium by means of the rolling-tube method, which makes it possible for cells to grow under strictly anaerobic conditions. The resulting colonies contained two or more kinds of cells. To isolate single colonies, ten single cells were picked up from cultures 138-3-1 and 183-1-1, by means of a micromanipulator, under anaerobic conditions and put immediately into K medium containing 1% cellobiose. After 2 to 3 days incubation, seven cells from 138-3-1 and five cells from 183-1-1 were found to grow on cellobiose at 60°C. These 12 cultures were transferred to K medium containing 1% cel-

lulose and incubated again at 60°C for 7 days. All seven cultures (138-A to G) from culture 138-3-1 produced ethaol at almost the same concentration (about 0.11 vol%). Two (183-A and B) of the five cultures (183-A to E) from culture 183-1-1 produced 0.12~0.15 vol% ethanol; the other three produced only 0.04 vol%. These results suggested that at least two kinds of cells were present in culture 183-1-1. One strain of culture 138-A was named strain No. 138, and one of culture 183-A was named strain No. 183.

Characterization of the isolates

The two isolates, strain Nos. 138 and 183, were observed under an electron microscope (Fig. 1). Both strains were rods with flagella. Morphological, physiological and genetic properties of the two isolates are shown in Table I, together with those of *C. thermocellum* ATCC 27405 for reference. The cell shape, spore formation, gram staining, optimum growth temperature, assimilation of carbohydrates, and main products of strain Nos. 138 and 183 were closely similar to those of the type stain, ATCC 27405. The GC content of the chromosomal DNAs isolated from the isolates was almost the same as that in the case of ATCC 27405. Furthermore, the chromosomal DNAs of the two isolates showed high homology

(98~100%) with the DNA of the type strain. From these results, strain Nos. 138 and 183 were identified as *C. thermocellum*.

Products from cellulose with the isolates

Strains Nos. 138 and 183 were grown at 60°C for 7 days in DSM medium containing 1% cellulose, with ATCC 27405 and ATCC 31549 for reference (Table II). Compared with the two type strains, these isolates decomposed more of the cellulose and produced large amounts of reducing sugars in the broth. The two isolates produced more ethanol, acetic acid and lactic acid than the type strains. Strain No. 183 in particular produced much lactic acid and glucose. When strain No. 138 was grown at 60°C for 7 days in K medium containing 6% cellulose in a jar fermentor (1.2 l medium/2 l jar), it produced 3.0 g/l of ethanol and consumed 75% of th cellulose added as the sole source of carbon.

Cellulase activities of th isolates

The CM-cellulase, avicelase and β -glucosidase activities of the two isolates and the type strain are shown in Table III. The CM-cellulase and avicelase activities in the culture broth of strain Nos. 138 and 183 were twice those in the case of ATCC 27405 when the cells were grown for 5 days in K medium containing

A **B**

Fig. 1. Electron Micrographs of Platinum-shadowed Cells of the Isolated Strains.

Bar, 1 μm. A, strain No. 138; B, strain No. 183.

Table I. MORPHOLOGICAL AND PHYSIOLOGICAL PROPERTIES OF THE ISOLATED STRAINS

Properties	Strain No.		
	138	183	ATCC 27405 ^a
Cell shape	Rods	Rods	Rods
Size of cells (μm)	$0.3 \times 1.5 \sim 4.5$	$0.3 \times 1.0 \sim 3.0$	$0.4 \times 1.0 \sim 2.0$
Spore formation	Terminal	Terminal	Terminal
Gram staining	Positive	Positive	Positive
Optimum temp.	60~63	63	60~64
Optimum pH	7.5~8.0	7.5~8.5	7.5~8.0
GC contents (mol%)	39.0	39.2	38.1
DNA homology to ATCC 27405	100	98	100
Fermentative sugars ^b	Fructose	Fructose	Fructose
	Glucose	Glucose	Glucose
	Cellobiose	Cellobiose	Cellobiose
	Cellulose	Cellulose	Cellulose
	Salicin	Salicin	Salicin
	Sorbitol	Sorbitol	Sorbitol
	Ethanol	Ethanol	Ethanol
	Acetate	Acetate	Acetate
	Lactate	Lactate	Lactate
Main products			

^a *C. thermocellum* ATCC 27405.^b Strain Nos. 138 and 183, and ATCC 27405 could not ferment the following sugars: arabinose, galactose, mannose, rhamnose, ribose, sorbose, xylose, lactose, maltose, melibiose, sucrose, melezitose, raffinose, trehalose, glycogen, inulin, starch, xylan, amygdalin, esculin, adonitol, dulcitol, glycerol, erythritol, inositol and mannitol.**Table II.** PRODUCTS FROM CELLULOSE WITH THE ISOLATED STRAINS

Strain No. ^a	Product (%)					Consumption of cellulose (%)
	Ethanol	Acetate	Lactate	Glucose	Reducing sugar	
138	0.076	0.096	0.055	0	0.695	96
183	0.047	0.083	0.103	0.220	0.860	81
ATCC 27405 ^b	0.030	0.024	0.022	0	0	14
ATCC 31549 ^b	0.023	0.023	0.027	0	0	14

^a The strains were grown at 60°C for 7 days in DSM medium containing 1% cellulose.^b *Clostridium thermocellum*.

1% cellulose as the sole source of carbon. On the other hand, the β -glucosidase activity in the cell free extracts of the two isolates was almost the same as that in the case of ATCC 27405. The production of CM-cellulase by strain Nos. 138 and 183, and ATCC 27405, on growth with cellobiose or glucose, was tested. As a result, it was found that they could produce CM-cellulase without inhibition by cellobiose or glucose.

Development of ethanol tolerance of the isolates

Strains Nos. 138 and 183, and ATCC 27405 were grown at 60°C for 2 to 3 days in DSM medium containing 1% cellobiose and various concentrations of ethanol. The two isolates and the type strain could grow in the medium with 1% ethanol. The three cultures were transferred sequentially to fresh media containing increasing concentrations of ethanol. After seven transfers, all three strains could grow in the presence of high concentrations (2.5 to 2.9%) of ethanol.

Table III. CELLULASE ACTIVITIES IN THE ISOLATED STRAINS

Strain No. ^a	Supernatant		Cell-free extract
	CM-Cellulase ^b	Avicelase ^b	β -Glucosidase ^b
138	5.99	1.63	2.86
183	4.21	1.38	2.43
ATCC 27405 ^c	2.64	0.78	2.30

^a The cells were grown in K medium containing 1% cellulose for 5 days.

^b Unit/mg of protein.

^c *C. thermocellum* ATCC 27405.

Cloning of the *celA* gene of No. 138

Chromosomal DNA extracted from *C. thermocellum* strain No. 138 was digested completely with *Hind*III. The DNA fragments obtained were electrophoresed on a 1% agarose gel and then hybridized with the two kinds of probe DNAs described above. Only a single DNA band (about 3.2 kb) in an agarose gel hybridized strongly with the probes. The 3.2 kb-DNA fragments were eluted from the gel and inserted into the same restriction site of plasmid pUC19. The resulting recombinant plasmids were introduced into *E. coli* strain JM83 cells. Of the plasmids extracted from approximately 800 ampicillin-resistant cells, two plasmids that hybridized strongly with the probe DNAs, designated as pNBC21 and pNBC22, were analyzed. The restriction maps of the *Hind*III fragments contained in the two plasmids were the same (Fig. 2). The restriction map of the *Hind*III fragment from strain No. 138 was also almost the same as that containing the *celA* gene from ATCC 27405. The *Hind*III fragment was inserted into the same site of plasmid pBR322 in the ordinary and opposite directions, and the recombinant plasmids obtained were designated as pNBC1 and pNBC2, respectively.

Expression of *celA* genes in *E. coli*

Recombinant plasmids pNBC1 and pNBC2 were introduced into *E. coli* strains C600 and HB101. The CM-cellulase activity of strain C600 cells carrying either pNBC1 or pNBC2

Fig. 2. Restriction Maps of the *Hind*III Fragments Containing the *celA* Gene from *Clostridium thermocellum* ATCC 27405 (a) and from Strain No. 138 (b).

The symbols for restriction endonucleases are: H, *Hind*III; S, *Sau*3AI; P, *Pst*I; and B, *Bam*HI. The molecular sizes (kb) of the linear DNA fragments were determined by agarose gel electrophoresis.

Table IV. CM-CELLULASE ACTIVITIES IN *E. coli* STRAINS CARRYING THE *celA* GENE

Strain ^a	Plasmid	<i>celA</i> gene	CM-Cellulase activity ^b
<i>E. coli</i> C600	—	—	0
C600	pBR322	—	0
C600	pCT104	+	5.52
C600	pNBC1	+	5.49
C600	pNBC2	+	5.07
HB101	—	—	0
HB101	pBR322	—	0
HB101	pCT104	+	6.7 ^c
HB101	pNBC1	+	6.69
HB101	pNBC2	+	7.98

^a Strains were grown at 37°C in L-broth. For the transformants, ampicillin (50 µg/ml) was added to the L-broth. Late log-phase cells were sonicated and the supernatants were used for assaying CM-cellulase.

^b Micromoles of glucose generated/hour/mg of protein.

^c The value reported by Cornet *et al.* (1983).

was almost the same as that of the cells carrying plasmid pCT104, in which the *celA* gene from ATCC 27405 was inserted into the *Hind*III site of pBR322 in the ordinary direction. This enzyme activity of strain HB101 carrying one of the plasmids was also the same as that of pCT104 (Table IV).

Discussion

To obtain cellulose-fermenting thermophiles, we isolated new strains of *Clostridium thermocellum*, Nos. 138 and 183, from soil and compost, respectively. When cellulolytic bacteria are isolated from nature, there are often coexisting strains, which cannot assimilate cellulose, around the colonies of the cellulolytic strains. We found a coexisting strain growing in culture 183-1-1. This strain could assimilate cellulose weakly. The two isolates, Nos. 138 and 183, which were purified by use of a micromanipulator under anaerobic conditions, were identified as strains of *C. thermocellum* because their morphological and physiological properties were the same as those of the type strain, ATCC 27405. Furthermore, the genetic properties of the two isolates, the GC contents of their chromosomal DNA, and their DNA homology to the chromosomal DNA of the type strain were evidence that they were *C. thermocellum* strains.

The two isolates, however, differed from the type strain in their ability to assimilate cellulose. They could decompose more cellulose than the type strains, ATCC 27405 and ATCC 31549, and had two times higher CM-cellulase and avicelase activities than the type strain had. The CM-cellulases of the two isolates were constitutively produced and they were also produced without being inhibited by cellobiose or glucose. These results suggested that the two isolates should be useful for the production of cellulases.

Cornet *et al.*⁸⁾ have cloned four cellulase genes from *C. thermocellum* ATCC 27405. Two, *celA* and *celB*, of the four genes have been studied in detail. They reported that the CM-cellulase activity of *E. coli* HB101 carrying the *celA* gene was higher than that of the same strain carrying the *celB* gene. The *celA* gene of strain No. 138 was cloned into *E. coli* with reference to the base sequence⁹⁾ of the *celA* gene from ATCC 27405. The restriction map of the *Hind*III fragment containing the *celA* gene from strain No. 138 showed good

agreement with that from ATCC 27405. Moreover, the expression of the two *celA* genes in *E. coli* cells was almost the same. These results also showed that strain No. 138 was a *C. thermocellum* strain. *E. coli* clones carrying plasmid DNA that had the *celA* gene inserted in the ordinary and opposite directions exhibited the same CM-cellulase activity; this suggested that a promoter of the *celA* gene from strain No. 138 acted in *E. coli* cells.

If the *celA* gene cloned was introduced into the original strain, No. 138, the CM-cellulase activity of the transformants could be increased. *C. thermocellum* transformants carrying high copy numbers of the *celA* gene might be of use for the production of ethanol from the cellulosic biomass.

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References

- 1) M. Taya, H. Hinoki, Y. Suzuki, T. Yagi, M. G. S. Yap and T. Kobayashi, *J. Ferment. Technol.*, **63**, 383 (1985).
- 2) J. Bender, Y. Vatcharapijarn and T. W. Jeffries, *Appl. Environ. Microbiol.*, **49**, 475 (1985).
- 3) D. Freier, C. P. Mothershed and J. Wiegel, *Appl. Environ. Microbiol.*, **54**, 204 (1988).
- 4) N. Ait, N. Creuzet and P. Forget, *J. Gen. Microbiol.*, **113**, 399 (1979).
- 5) E. A. Johnson, F. Bouchot and A. L. Demain, *J. Gen. Microbiol.*, **131**, 2303 (1985).
- 6) D. Petre, J. Millet, R. Longin, P. Beguin, H. Girard and J.-P. Aubert, *Biochimie*, **68**, 687 (1986).
- 7) P. Cornet, D. Tronik, J. Millet and J.-P. Aubert, *FEMS Microbiol. Lett.*, **16**, 137 (1983).
- 8) P. Cornet, J. Millet, P. Beguin and J.-P. Aubert, *Bio/Technol.*, **1**, 589 (1983).
- 9) P. Beguin, P. Cornet and J.-P. Aubert, *J. Bacteriol.*, **162**, 102 (1985).
- 10) D. V. Garcia-Martinez, A. Shinmyo, A. Madia and A. L. Demain, *Eur. J. Appl. Microbiol. Biotechnol.*, **9**, 189 (1980).
- 11) J. N. Saddler and K. H. Chen, *Eur. J. Appl.*

- Microbiol. Biotechnol.*, **16**, 99 (1982).
- 12) N. Kurose, S. Kinoshita, J. Yágyu, M. Uchida, S. Hanai and A. Obayashi, *J. Ferment. Technol.*, **66**, 467 (1988).
- 13) G. L. Miller, *Anal. Chem.*, **31**, 426 (1959).
- 14) H. C. Birnboim and J. Doly, *Nucl. Acids Res.*, **7**, 1513 (1979).
- 15) N. Nelson, *J. Biol. Chem.*, **153**, 375 (1944).
- 16) M. M. Bradford, *Anal. Chem.*, **72**, 248 (1976).
- 17) O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
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