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Characterization of 13 newly isolated strains of anaerobic, cellulolytic, thermophilic bacteria

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Characteristics of 13 newly isolated thermophilic, anaerobic, and cellulolytic strains were compared with previously described strains of *Clostridium thermocellum*: ATCC 27405 and JW20 (ATCC 31549). Colony morphology, antibiotic sensitivity, fermentation end-products, and cellulose degradation were documented. All 13 strains were sensitive to erythromycin (5 μg/ml) and chloramphenicol (25 μg/ml), and all strains but one were sensitive to kanamycin (20 μg/ml). Polymerase chain reaction (PCR) amplification using primers based on gene sequences from *C. thermocellum* ATCC 27405 was successful for all 13 strains in the case of the hydrogenase gene and 11 strains in the case of phosphotransacetylase/acetate kinase genes. Ten strains amplified a product of the expected size with primers developed to be specific for *C. thermocellum* 16SrRNA primers. Two of the 13 strains did not amplify any product with the PCR primers designed for the phosphotransacetylase/acetate kinase and 16SrRNA primers. A *MboI*-like GATC-recognizing restriction activity was present in all of the five strains examined. The results of this study have several positive implications with respect to future development of a transformation system for cellulolytic thermophiles. *Journal of Industrial Microbiology & Biotechnology* (2001) **27,** 275–280.

Keywords: cellulolytic; anaerobic; thermophilic; *Clostridium thermocellum*; 16SrRNA

Introduction

Previously described species of anaerobic, cellulolytic, moderately thermophilic (optimal growth temperature ~60°C) bacteria include *Clostridium thermocellum* [16], *C. stercorarium* [12], *C. thermolacticum* [9], *C. thermocapriae* [7], and *C. thermopapyrolyticum* [13]. All of these Gram-positive species form spores and produce ethanol and/or organic acids as fermentation products.

C. thermocellum is the most thoroughly described bacterium of the anaerobic cellulolytic thermophiles and exhibits a high growth rate on cellulose relative to mesophilic, cellulolytic bacteria [16]. Because of these features, *C. thermocellum* represents a departure point for developing microorganisms capable of "consolidated bioprocessing" (CBP), whereby cellulase production, cellulose hydrolysis, and production of a desired product are accomplished in a single process step [10]. For *C. thermocellum* and similar organisms to be useful for ethanol production *via* CBP, it is desirable to manipulate the end-product metabolism so that ethanol is produced with a high selectivity relative to organic acids. In *C. thermocellum* ATCC 27405, acetate is the primary organic acid produced, with smaller amounts of lactate produced under some conditions. Recently, we have cloned and sequenced a putative acetate kinase/phosphotransacetylase operon (GenBank accession no. AF041841) and a hydrogenase gene (GenBank accession no. AF148212) originating from *C. thermocellum* 27405. Thus, these genes are available for use in studies featuring homologous recombination-mediated gene knockout, and there are no apparent methodological challenges to obtaining additional gene clones as needed. The key impediment to such studies at this time is the absence of a well-established gene transfer system for a cellulolytic thermophile.

Screening a variety of strains is one useful strategy in developing new transformation systems, as the mechanistic determinants of transformability are, in general, obscure. For example, Narumi *et al* [15] screened 62 strains of *Bacillus stearothermophilus* in order to obtain a strain that was readily transformable by electroporation. Rawlings and Kusano [19] screened 30 strains of *Thiobacillus ferrooxidans* to find one that was electrocompetent. In work that has not been followed up to our knowledge, Tsoi *et al* [21] screened six strains identified as *C. thermocellum*, one of which was shown to be amenable to protoplast transformation. A second important factor in developing transformation systems is restriction endonuclease activity of potential DNA recipients as well as sequence-specific methylases that protect DNA from restriction attack. Mermelstein and Papoutsakis [15] found that electroporation frequencies of *C. acetobutylicum* were reduced by about one order of magnitude per restriction site on non-methylated plasmids. Native restriction systems of several *Clostridium* species have been reported [1,2,4,20,23]. Previously we have found that the restriction enzyme recognition sequence for *C. thermocellum* ATCC 27405 is 5′-GATC-3′, and that the *dam* methylation system of *Escherichia coli* protected plasmids from digestion in *C. thermocellum* [8].

In this study, we isolated 13 strains of cellulolytic thermophiles. These strains, as well as *C. thermocellum* strains ATCC 27405 and JW20, are compared with respect to the extent of cellulose hydrolysis and fermentation end-products formed, colony morphology, sporulation, antibiotic resistance, and their

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Table 1 General characterization of the newly isolated strains

Strains	Source	Colony morphology	Cellulose hydrolyzed (g/l)	Glucose (g/l)	Ethanol (g/l)	Acetate (g/l)	Lactate (g/l)	Carbon balance (%)	pH	Clearing zone, colony size (mm)	
1.1.1	Lawn soil	yellow, shiny	9.20±0.75	ND	2.30±0.17	2.46±0.32	ND	89	5.21	1.0	0.2
2.12.1	Rotten garden mulch	pale yellow, small	5.08±0.45	ND	0.73±0.15	1.844±0.31	0.09±0.02	84	5.59	1.1	0.5
6.3.2*	Lawn compost	pale yellow, small	7.77±0.63	ND	1.32±0.22	1.21±0.21	2.05±0.22	83	5.49	1.0	0.1
7.1.2	Composted sheep dung	pale yellow, shiny, slimy	8.55±0.21	ND	2.77±0.19	0.59±0.15	1.02±0.11	85	6.30	2.8	0.8
7.7.10*	Composted horse dung	pale yellow, shiny, small	8.39±0.38	1.80±0.23	1.29±0.08	1.27±0.15	0.82±0.14	79	5.39	0.5	0.1
7.8.3	Composted horse dung	Transparent	7.36±0.26	ND	1.28±0.02	2.34±0.21	ND	82	5.78	1.1	0.3
7.9.1*	Composted horse dung	pale yellow, normal size	8.75±0.75	2.59±0.36	0.98±0.11	2.08±0.35	0.29±0.03	86	5.47	3.1	0.5
7.9.4	Sheep dung	pale yellow, small	7.58±0.61	1.57±0.12	0.82±0.12	0.61±0.11	2.13±0.21	77	5.88	0.6	0.1
7.10.1	Composted cow dung	pale yellow, small	9.11±0.74	ND	3.31±0.63	1.02±0.21	0.30±0.05	91	6.07	0.5	0.1
7.10.4	Composted cow dung	pale yellow	8.97±0.40	ND	1.57±0.11	2.11±0.22	0.90±0.12	79	5.21	1.0	0.4
7.12.1	Composted cow dung	transparent	9.07±0.92	ND	2.03±0.16	2.41±0.16	ND	83	5.8		
11.8.1	Dartmouth compost	yellow, shiny	8.81±0.39	ND	2.66±0.16	1.03±0.14	ND	76	5.29	0.5	0.1
12.8.1	Dartmouth compost	transparent, small	4.56±0.55	ND	1.049±0.06	1.23±0.09	ND	85	5.82	No zone	No zone
C. thermocellum JW20	J. Wiegel	–	10.24±0.27	ND	4.05±0.17	1.00±0.14	0.20±0.03	94	5.15	–	–
C. thermocellum 27405	ATCC	–	10.05±0.36	ND	4.32±0.58	0.76±0.07	ND	95	5.5	5.0	16.0

*Additional unknown peaks observed.

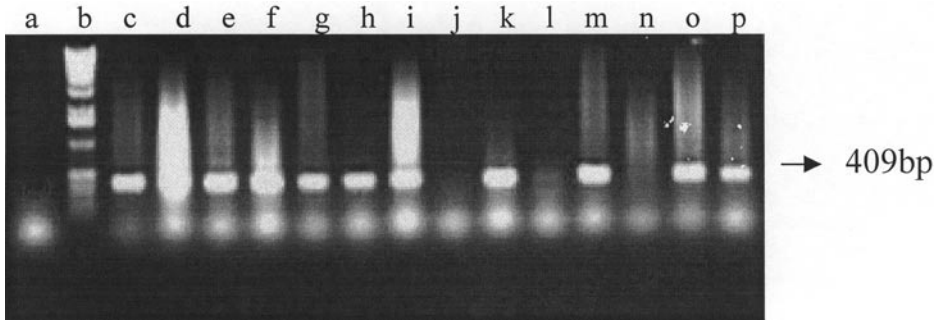

Figure 1 PCR amplification using primers for 16srRNA gene: a — No DNA; b — DNA Marker; c — *C. thermocellum*; d — 1.1.1; e — 2.12.1; f — 6.3.2; g — 7.1.2; h — 7.7.10; i — 7.8.3; j — 7.9.1; k — 7.9.4; l — 7.10.1; m — 7.10.4; n — 7.12.1; o — 11.8.1; p — 12.8.1.

phylogenetic relatedness to *C. thermocellum* using PCR probes for 16SrRNA [5], hydrogenase, and phosphotransacetylase/acetate kinase genes. Several of these strains were also examined for the presence of sequence-specific restriction endonuclease activity.

Materials and methods

Strains, plasmids, and chemicals

C. thermocellum JW20 was a gift from J. Wiegel (University of Georgia, Athens, GA). *C. thermocellum* 27405 (same as ATCC 27405) was a gift from A. Demain (MIT, Cambridge, MA). *E. coli* strains ED8767 (*dam*+) and DH5α (*dam*−) were gifts from New England Biolabs (Beverly, MA). Plasmid pHV33 was a gift from P. Vary (Northern Illinois University, Dekalb, IL) [4]. Bacterial strains were maintained either in the medium described by Tsoi *et al* [21], designated as RM in this study, or MTC with 0.5% avicel or cellobiose as the carbon source. RM medium contained 2 g/l urea, 2 g/l KH_2PO_4, 3 g/l K_2HPO_4, 5 g/l yeast extract, 0.2 g/l $MgCl_2{\cdot}6H_2O$, 0.05 g/l $CaCl_2{\cdot}2H_2O$, 0.0025 g/l $FeSO_4{\cdot}7H_2O$, 1 g/l L-cysteine. MTC medium contained 5.0 g/l MOPS, 5.0 g/l yeast extract, 5.0 g/l β-glyceromonophosphate, 1 ml of 1 g/l rasazurin, 2% agar, 2.0 g/l citric acid ($C_6H_5O_7K_3$), 1.25 g/l citric acid monohydrate ($C_6H_8O_7{\cdot}H_2O$), 1.0 g/l sodium sulfate, 1.0 g/l potassium phosphate (KH_2PO_4), 2.5 g/l sodium bicarbonate $NaHCO_3$, 1.5 g/l NH_4Cl, 1.0 g/l $MgCl_2{\cdot}6H_2O$, 0.2 g/l $CaCl_2{\cdot}2H_2O$, 0.1 g/l $FeCl_2{\cdot}4H_2O$, 1.0 g/l L-cysteine hydrochloride monohydrate ($C_3H_7NO_2S{\cdot}HCl{\cdot}H_2O$), 20 mg/l prydoxamine dihydrochloride, 4 mg/l *p*-aminobenzoic acid (PABA), 4 mg/l D-biotin, 2 mg/l vitamin B_{12}, 4 mg/l thiamine, 1.0 g/l $MgCl_2{\cdot}6H_2O$, 0.2 g/l $CaCl_2{\cdot}2H_2O$, 0.1 g/l $FeCl_2{\cdot}4H_2O$, 0.5 mg/l $MnCl_2{\cdot}4H_2O$, 0.5 mg/l $CoCl_2{\cdot}6H_2O$, 0.2 mg/l $ZnCl_2$, 0.05 mg/l $CuCl_2{\cdot}2H_2O$, 0.05 mg/l H_3BO_3, 0.05 mg/l $Na_2MoO_4{\cdot}2H_2O$, 0.05 mg/l $NiCl_2{\cdot}6H_2O$, 1.0 g/l L-cysteine hydrochloride monohydrate ($C_3H_7NO_2S{\cdot}HCl{\cdot}H_2O$). Medium was prepared in an anaerobic chamber with an atmosphere of $CO_2/N_2/H_2$ (10%/85%/5%). Thermophilic cultures were incubated at 55°C and *E. coli* strains were grown on Luria–Bertani media at 37°C. All medium chemicals were from Sigma (St. Louis, MO) unless otherwise noted and restriction enzymes were from New England Biolabs (Beverly, MA) or Gibco BRL (Rockville, MD).

Isolation of cellulolytic strains

Samples were collected from various environments (Table 1) and grown in RM without yeast extract, but supplemented with minimal salts. Avicel (FMC Philadelphia, PACAS no. 51395-75-6), a microcrystalline cellulose, was used as the carbon source for enriching all the environmental samples. Cultures were grown at 55°C in glass tubes sealed under nitrogen. The cultures were transferred four times to ensure that they were able to utilize avicel as a carbon source and were not living solely on nutrients in the environmental sample. Thereafter, bacteria from the environmental samples were maintained in broth tubes as mixed cultures and plated out on anaerobic Hungate roll tubes containing the appropriate media and avicel. Individual colonies were picked from the roll tubes and inoculated in broth to obtain environmental isolates. Pure cultures of cellulose degraders were stored as spore at room temperature and the spore stocks were prepared as indicated below.

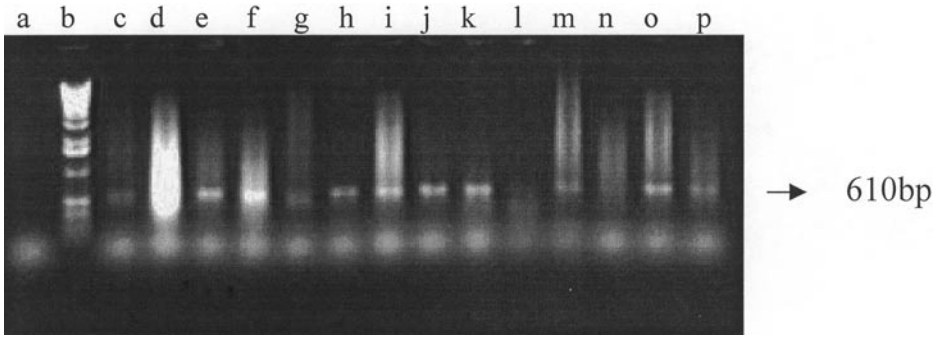

Figure 2 PCR amplification using primers for phosphotransacetylase/acetate kinase gene. Lanes are as shown for Figure 1.

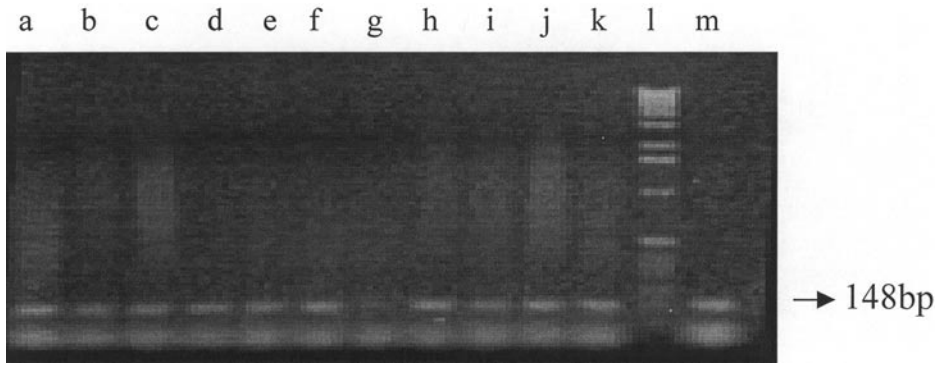

Figure 3 PCR amplification using primers for hydrogenase gene: a — 6.3.2; b — 7.1.2; c — 7.7.10; d — 7.8.3; e — 7.9.1; f — 7.9.4; g — 7.10.1; h — 7.10.4; i — 7.12.1; j — 11.8.1; k — 12.8.1; l — DNA Marker; m — *C. thermocellum*.

Sporulation test

Spore stocks of cellulolytic isolates were prepared as follows: 1 cm^2 sterilized Whatman no. 1 filter papers were absorbed with the sporulated cultures of environmental isolates and filter papers were dried completely in sterile Petri dishes in aerobic conditions. They were stored for three mounts and used for inoculation of fresh medium. Cultures were grown for 10 days in order to see growth by regeneration of spores.

Antibiotic resistance test

Antibiotics were added to the cellobiose RM media in the following concentrations: erythromycin, 5 μg/ml; kanamycin, 5, 10, 20, 40 μg/ml; and chloramphenicol, 25 μg/ml. Inoculated medium was incubated for 7–10 days. Measuring OD at 660 nm determined growth.

Determination of cellulose degradation zones

MTC medium with 2% agar and 0.5% avicel as carbon source prepared in Hungate roll tubes was inoculated with 100 μl culture and solidified using a tube spinner at 4°C. After 15 days of incubation, the diameter of the degradation zones was measured using millimeter graph paper.

Determination of fermentation end-products and cellulose degradation rates

Cultures of cellulolytic isolates were grown in RM broth with 1% avicel in anaerobic culture tubes. Samples were taken after 4–5 days of growth and were assayed using HPLC after centrifuging the cell culture [11]. The residual cellulose was measured by quantitative saccharification [11]. Carbon balance was expressed as percentage of [2(moles ethanol+moles acetate+moles lactate)/(moles total cellulose used)].

Comparison of hydrogenase, phosphotransacetylase/acetate kinase, and 16SrRNA gene sequences by PCR

Primers designed for an internal region of hydrogenase, phosphotransacetylase/acetate kinase, and 16SrRNA of *C. thermocellum* ATCC 27405 were synthesized by Molecular Biology Core Facilities (Dartmouth College, Hanover, NH). Primers for amplifying a 610-bp region of *pta/ack* gene were 5′-GAA AAT CGA AGG GTA TGA CTA TA-3′ and 5′-TTT GCC AAT CTC TGT GTA AGC-3′. Primers for amplifying a 148-bp region of hydrogenase gene were 5′-GGT TTT GAA ATC CTA CTA TGC-3′ and 5′-TAG AAC AAC ATC CAC ATC AGG-3′. Primers for amplifying a 148 bp region of hydrogenase gene were 5′-GGT TTT GAA ATC CTA CTA TGC-3′ and 5′-TAG AAC AAC ATC CAC ATC AGG-3′. Primers for 16SrRNA were as described by Erbeznik *et al* [6].

Restriction endonuclease characterization

Cell extracts of stationary phase cells were prepared by passing the cells through a French pressure cell at 4°C. Extracts were stored at −20°C. DNA digests were prepared by combining 0.4 μg of plasmid DNA extracted from either *dam*+ or *dam*− strains of

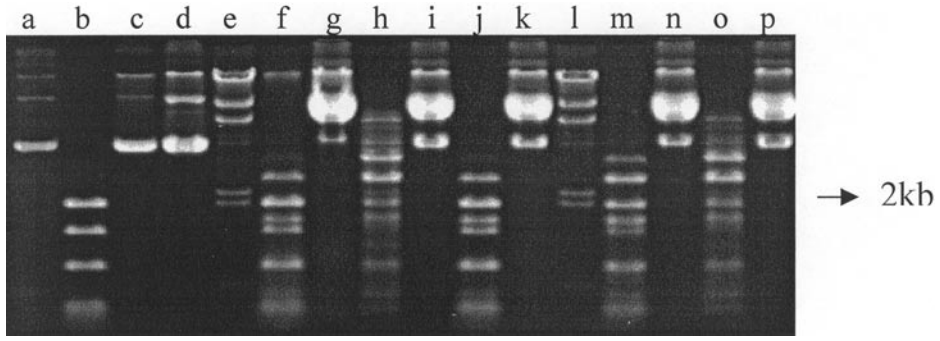

Figure 4 *MboI*-like, GATC-recognizing restriction activity: Methylated (+) and non-methylated (−) plasmid pHV33 digested with *DpnII*. Lane h, m and o might be partial digests. a — uncut pHV33 (−); b — cut pHV33 (−); c — uncut pHV33 (+); d — cut pHV33 (+); e — Marker; f — *C. thermocellum* (−); g — *C. thermocellum* (+); h — JW20 (−); i — JW20 (+); j — 2.12.1 (−); k — 2.12.1 (+); l — Marker; m — 6.3.2 (−); n — 6.3.2 (+); o — 11.8.1 (−); p — 11.8.1 (+).

E. coli, 1 μl of cell or protoplast extract, and 1 μl of 10× high salt buffer, which contains 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT (pH 7.9 at 25°C) in a final volume of 10 μl. The mixture was incubated at 55°C. Methylated and non-methylated pHV33 DNA was digested with *DpnII* at 37°C for 3 h.

Results and discussion

The sources of the 13 cellulolytic strains are listed in Table 1 with most of them originating from soil or compost. All exhibited rod-shaped morphology, although with variable size, and spore stocks of all the strains were found to be viable. All 13 strains were sensitive to chloramphenicol (25 μg/ml) and erythromycin (5 μg/ml), and except for strain 7.10.1 all strains were sensitive to kanamycin (20 μg/ml) in liquid cultures.

Results for fermentation end-products, final pH, and cellulose degradation are presented in Table 1. Eleven of the 13 strains hydrolyzed cellulose to an extent comparable with the previously described strains *C. thermocellum* ATCC 27405 and JW20. Strains 2.12.1 and 12.8.1 were relatively poor Avicel degraders. Ethanol, acetate, and lactate were the main end-products in most of the strains; ethanol was produced by all the strains and was the primary product in three of the 13 strains (7.1.2, 7.10.1, and 11.8.1), measured on a g/L basis. Isolates 7.8.3, 7.9.1, and 7.10.4 degraded cellulose well and produced acetic acid as the main product while two other strains (6.3.2 and 7.9.4) produced mainly lactate. Two strains (1.1.1 and 7.12.1) produced roughly equal amounts of ethanol and acetic acid as the primary products. Isolate 7.7.10 produced ethanol, acetate, and lactate in comparable proportions. The variable ratio of end-products formed by the isolates found in our study is in contrast to the results of Bender *et al* [3] who reported a constant ratio of final moles of acetic acid to ethanol formed in six of six isolates examined. The pH of the cultures was set to 7.0 at the beginning and the pH of the medium decreased with increasing product formation to final values in the range of 5.15–6.3. The colony and clearing zone size of the 13 isolates, as well as *C. thermocellum* ATCC 27405 and JW20, did not correlate with the amount of cellulose hydrolyzed in liquid culture.

In 10 of the 13 strains, but not for three others, PCR amplification was observed with primers specific to *C. thermocellum* 16SrRNA as shown in Figure 1. 16SrRNA gene sequence is used to determine phylogenetic relationship between different species of bacteria [17,18]. Thus, the three strains that did not show the expected 409-bp band with 16SrRNA primers may be different from *C. thermocellum*. The PCR primers were originally designed for the purpose of distinguishing *C. thermocellum* from *Thermoanaerobacter* strains [5]. Hence, the 10 strains that showed bands of 409-bp size are different from the genus *Thermoanaerobacter*. As seen in Figure 2, all but two strains (7.10.1 and 7.12.1) amplified a product of the expected size of 610 bp with primers designed for the phosphotransacetylase/acetate kinase genes from *C. thermocellum*. The same two strains did not amplify the expected product with 16SrRNA primers, further suggesting that these strains are more distantly related to *C. thermocellum*. PCR amplification was observed for all 13 strains using primers designed for the hydrogenase gene from *C. thermocellum* (Figure 3).

Figure 4 shows the digestion pattern of *dam*-methylated and non-methylated plasmid pHV33 using the restriction activity of protoplasted cell extracts for five strains. *DpnII* cuts only non-methylated DNA at GATC. *MboI*-like, GATC-recognizing restriction activity, as shown by *C. thermocellum* ATCC 27405, was observed in *C. thermocellum* JW20 and all of the three new isolates examined, 2.12.1, 6.3.2, and 11.8.1. Additional bands seen in some lanes are believed to be due to partial digestion of plasmid pHV33. The results shown in Figure 4 suggest that the presence of a GATC-recognizing *dam*-protected restriction endonuclease may be characteristic of cellulolytic thermophiles.

The results of this study have several positive implications with respect to future development of a transformation system for cellulolytic thermophiles, including: (a) documentation of widespread sensitivity of cellulolytic thermophiles to several antibiotics; (b) demonstration that DNA prepared in dam^+ hosts is resistant to endonuclease attack in many strains; and (c) availability of a substantial number of ethanol-producing strains that can be used to screen for transformation competency.

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