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Isolation and Characterization of *Shigella flexneri* G3, Capable of Effective Cellulosic Saccharification under Mesophilic Conditions▽†

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A novel *Shigella* strain (*Shigella flexneri* G3) showing high cellulolytic activity under mesophilic, anaerobic conditions was isolated and characterized. The bacterium is Gram negative, short rod shaped, and nonmotile and displays effective production of glucose, cellobiose, and other oligosaccharides from cellulose (Avicel PH-101) under optimal conditions (40°C and pH 6.5). Approximately 75% of the cellulose was hydrolyzed in modified ATCC 1191 medium containing 0.3% cellulose, and the oligosaccharide production yield and specific production rate reached 375 mg g Avicel^{-1} and 6.25 mg g Avicel^{-1} h^{-1}, respectively, after a 60-hour incubation. To our knowledge, this represents the highest oligosaccharide yield and specific rate from cellulose for mesophilic bacterial monocultures reported so far. The results demonstrate that *S. flexneri* G3 is capable of rapid conversion of cellulose to oligosaccharides, with potential biofuel applications under mesophilic conditions.

Lignocellulosic biomass is abundant in nature, as well as in agricultural, forestry, and municipal wastes, and can be used as an excellent bioconversion feedstock. Biomass-derived saccharides, such as glucose, cellobiose, and other minor sugars, can be readily fermented by appropriate microbes into bioenergy products, such as hydrogen, ethanol, biodiesel, and other commodity chemicals. However, the high cost of converting biomass to sugars is the primary factor impeding establishment of a cellulosic-biofuels industry (24, 40). Lignocellulosic biofuels can be competitive on an industrial scale if efficient technologies can be developed (29, 39). Currently, the most efficient process for utilization of cellulose as a feed stock is either a three-step process (separate hydrolysis and fermentation [SHF]) involving separate pretreatment, cellulose hydrolysis (i.e., saccharification), and hexose and pentose fermentation steps or a two-step process (simultaneous saccharification and fermentation [SSF]) involving separate pretreatment and simultaneous saccharification of hexose and pentose fermentation (48, 64). Combining hydrolysis of cellulose with simultaneous fermentation of hexose and pentose in a single process, i.e., direct microbial conversion (DMC), is an ideal strategy for converting cellulosic biomass to ethanol. However, no single microorganism/community can implement DMC with high efficiency (67). In any of these configurations, rapid and efficient saccharification is critical for developing competitive biotechnologies for cellulosic-biofuel production.

In nature, cellulose is hydrolyzed to oligosaccharides by microorganisms, mainly fungi (e.g., brown-, white-, and soft-rot fungi) and bacteria (e.g., *Clostridium* and *Cellulomonas*), which produce either free cellulolytic enzymes or extracellular enzyme complexes known as cellulosomes (12). Many white-rot *Basidiomycetes* and some *Actinomycetes* have been employed for hydrolyzing lignocellulosic materials. For example, *Trichoderma reesei* has shown the highest cellulolytic activity currently known (27). Alternatively, anaerobic bacteria, such as *Clostridium thermocellum*, can also be used for biomass conversion. Recent studies have shown that certain thermophilic anaerobes, such as *C. thermocellum* strains, have sufficiently high growth and metabolic rates when grown on cellulose to make them competitive with fungi (14, 32, 34, 36, 45, 55). Many of these anaerobic bacteria utilize high-affinity cellulases organized in stable, membrane-bound, multienzyme cellulosomes, which greatly improve the efficiency of biomass conversion compared to fungi (46, 51). Anaerobic processes also have advantages over aerobic processes in terms of cost, incubation maintenance, and implementation for simultaneous saccharification and fermentation schemes (5, 47).

Although thermophilic bacteria are attractive for many industrial processes that are carried out at high temperatures, mesophilic organisms are important for many industrial processes typically carried out at room temperature. These processes include bioremediation by anaerobic fermentation of wastes for hydrogen, methane, and ethanol production, as well as biocatalytic and biochemical processes for industrial waste treatments (42). In particular, mesophilic microorganisms producing ethanol at high yield from sugars other than glucose present in biomass have been developed by increasing ethanol yields in bacteria (53). However, cellulose hydrolysis under mesophilic conditions is generally slow and inefficient. Thus, the goal of this study was to isolate bacterial strains capable of

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rapid production of high yields of oligosaccharides from lignocellulosic biomass under mesophilic conditions. Here, we report the isolation and characterization of a new bacterial strain, *Shigella flexneri* strain G3, capable of rapid and efficient production of sugars from cellulose (e.g., Avicel) under mesophilic conditions. Our results suggest that *S. flexneri* strain G3 could be an attractive candidate for converting cellulose to sugars under mesophilic conditions.

MATERIALS AND METHODS

Strain isolation and growth. Rumen fluid samples were collected at a slaughterhouse in Harbin, China, for enrichment culturing as previously reported (61). Briefly, the fluid was filtered through four layers of gauze and stored in a vial purged with N_2 gas prior to use. The filtered rumen fluid (10% [vol/vol]) was added as an inoculum to 50 ml Mf medium (modified from ATCC 1191 medium with filter paper as a substrate instead of glucose [filter strip, 1 by 8 cm, approximately 0.05 g, grade 1; Whatman]) and incubated without shaking at 37°C in a 100-ml top-sealed bottle flask. After a 5-day incubation, the resultant culture broth was added to fresh Mf medium (10% vol/vol) and cultured for another 5 days. After this enrichment process was repeated three times, the culture broth was placed on solid Mp medium for pure-culture isolation. The Mp medium (modified from ATCC 1191 medium) was used for bacterial isolation, containing (per liter) 5.0 g of Avicel PH-101 (50 μM; Huka Biochemika 11365; Sigma-Aldrich Chemie), 1.5 g of KH_2PO_4, 4.2 g of $Na_2HPO_4 \cdot 12H_2O$, 0.5 g of NH_4Cl, 0.18 g of $MgCl_2 \cdot 6H_2O$, 1.0 g of yeast extract (YE), 0.5 g of L-cysteine, and 1 ml of resazurin (0.2%). Agar was added (1.5% [wt/vol]) for the preparation of the solid medium. The medium was supplemented with 0.5 ml liter^{-1} vitamin solution containing 40.0 mg of biotin, 100.0 mg of *p*-aminobenzoic acid, 40 mg of folic acid, 100.0 mg of pantothenic acid calcium salt, 2 mg of B_{12}, 10.0 mg of thiamine-HCl, 200.0 mg of pyridoxine hydrochloride, 100.0 mg of thioctic acid, 10.0 mg of riboflavin, and 5.0 ml liter^{-1} micronutrients containing 1.5 g of nitrilotriacetic acid, 3.0 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot H_2O$, 1.0 g of NaCl, 0.1 g of $FeSO_4 \cdot 7H_2O$, 0.1 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.1 g of $CaCl_2$ (anhydrous), 0.1 g of $ZnSO_4 \cdot 7H_2O$, 0.1 g of $CuSO_4 \cdot 5H_2O$, 0.01 g of $Al \cdot K(SO_4)_2$ (anhydrous), 0.01 g of boric acid, 0.01 g of $Na_2MnO_4 \cdot 2H_2O$, and 0.001 g of Na_2SeO_3 (anhydrous). The enrichment, isolation operation, and cultivation were performed in a Vinyl Type A anaerobic chamber (Coy Laboratory Products, Inc.) containing an 80% N_2-20% CO_2 atmosphere and operated at 37°C. Representative colonies grown on 0.5% (vol/vol) Avicel were selected after a 4-day incubation. This process was repeated until the culture produced a uniform microbial colony. Colonies with active cellulase activity were identified by the clear zone encircling the colony.

The physiological characteristics of the selected isolate were determined by using API 50CH and ZYM test kits (bioMérieux, VWR, Inc.) as described by the manufacturer. Inoculated cultures were incubated at 37°C, and the color development was read after 24 and 48 h. Batch tests without pH control were conducted with 80 ml medium in 100-ml flasks in a shaker at 130 rpm at 37°C. Logarithmic-growth-phase cultures grown in the same medium were used as the inoculum (10% [vol/vol]). The cells were imaged with an Axioskop 2 Plus optical microscope (Zeiss, Oberkochen, Germany) and a scanning electron microscope (mode 3500N; Hitachi, Tokyo, Japan) according to the manufacturer's instructions. The specimen for scanning electron microscopy was prepared by the osmium fixation protocol and critical-point drying (56).

Strain identification. Genomic DNA was extracted using a Bacterial DNA Mini Kit (Watson Biotechnologies Co. Ltd., Shanghai, China) according to the manufacturer's instructions. The extracted DNA was used as the template for PCR amplification of the 16S rRNA gene. The 16S rRNA gene was amplified with a pair of universal primers: BSF8/20, 5′-AGAGTTTGATCCTGGCTCAG-3′, and PLA, 5′-GGTACTTAGATGTTTCAGTTC-3′ (Invitrogen, Co., Ltd., Shanghai, China) (49). The reaction mixture (50 μl) contained 10× PCR buffer, 10 mmol/liter Tris-HCl, 0.2 mmol/liter deoxynucleoside triphosphate (dNTP), 2.5 U of *Taq* DNA polymerase, 0.5 μmol/liter forward primer and 0.5 μmol/liter reverse primer, and 20 ng template DNA. The samples were amplified using a 9700PCR meter (Bio-Rad Laboratories, Hercules, CA) with the following thermal profile: 95°C for 4 min and 35 cycles of 40 s at 95°C, 40 s at 55°C, and 1 min at 72°C with a 0.1°C decrease in annealing temperature per cycle to 58°C. The PCR-amplified 16S rRNA was purified, and its size was verified by low-melting-point agarose electrophoresis.

Sequencing was performed at the Oklahoma Medical Research Foundation (http://omrf.org/). The nucleotide sequences were compared with the sequences in the GenBank/EMBL/DDBJ nucleotide sequence databases by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/) (2) and the Sequence Match program at the Ribosomal Database Project (RDP) (8). Alignment was carried out using ClustalX (59). Phylogenetic dendrograms were reconstructed using the MEGA program (version 4.0) (58) with the neighbor-joining (NJ) algorithm and bootstrap analysis (13, 58) of 1,000 replicates.

Growth optimization. To optimize the conditions for cellulolytic saccharification, strain G3 was tested in batch culture in Mp liquid medium for the following properties: (i) optimal pH and temperature conditions and (ii) effects of substrate and yeast extract concentrations. The original pH of the Mp liquid medium was adjusted, using sterile 1 N H_2SO_4 or 1 N NaOH, from 4.0 to 8.0 at intervals of 1.0 prior to inoculation; the tested temperature was changed from 20 to 45°C at 5°C intervals. Yeast extract concentrations ranged from 0.5 g liter^{-1} to 3.5 g liter^{-1}, and Avicel was added at eight concentrations: 1.0, 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, and 6.0 g liter^{-1}. Samples were taken every 5 h for a 100-h incubation period to determine the cell biomass, pH change, cellulase activity, saccharide yield, and concentrations of end products.

Total protein content. Biomass was estimated by measuring the total bacterial-protein content after cell lysis pretreatment. The process was as follows. One milliliter of mid-log-phase bacterial-cell culture with Avicel was collected by centrifugation at the maximum speed (14,000 × *g*) for 10 min at room temperature. After the suspension was discarded, 300 μl of the mixture (0.303 g of Tris, 0.189 ml of HCl, 0.8 g of SDS dissolved in 10 ml of ultrapure water) was added to the pellet and incubated at 100°C for 20 min for cell lysis. After cooling, the supernatant was collected by centrifugation at the maximum speed (14,000 × *g*) at room temperature and used to determine the cellular protein content using the Bradford method (4). A standard curve was generated using a series of concentrations of bovine serum albumin (BSA) solution as standards. Absorbance was measured in triplicate at 595 nm after 2 min of incubation at room temperature.

Analysis of cellulose and hydrolyzed products. The cellulose concentration was determined using the method of Huang et al. (26). Residual cellulose was washed using acetic acid-nitric acid reagent and water to remove noncellulosic materials as described by Updegraff (60). The cellulose was then quantified using the phenol-sulfuric acid method (11) with glucose as the standard. The relative crystallinity index (CI) of the cellulose was determined as described by Kim and Holtzapple (30), followed by removing adherent microbial cells. A Hewlett-Packard high-performance liquid chromatography (HPLC) chromatograph (HP1090) equipped with a refraction index detector (RID-10A; Shimadzu, Japan) and a chromatopac data processor (C-R8A; Shimadzu, Japan) system was used to detect soluble saccharides (specific to cellobiose and glucose), and one with a UV detector at 210 nm was used to detect volatile fatty acid (VFA) in the filtered (0.2 mm) supernatant of culture broth. HPLC-grade pure standards of soluble sugars (glucose, cellobiose, cellotriose, and cellotetraos) and volatile fatty acids (acetate, propionate, butyrate, and lactate) purchased from Fisher Scientific Co. (Fair Lawn, NJ) were used as internal standards in the analysis. Different concentrations of the standards were used based on the range required to plot a reliable calibration curve. The Avicel hydrolysis ratio (percent) was presumed to be the fraction of the cellulose that was consumed given as a percentage of the total provided as a substrate. Oligosaccharide yields were calculated as the amount of oligosaccharides produced (mg) per gram of Avicel added. Specific oligosaccharide production rates were calculated as the amount of oligosaccharides produced (mg) per gram of Avicel added per hour. An Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) was used to separate end products with 4 mM H_2SO_4 as the mobile phase.

Cellulase activity. The total cellulase activity was based on the Avicelase determination method described by Wood and Bhat (66). Endoglucanase activity was determined against carboxymethyl cellulose (CMC) (7), followed by reducing sugar (RS) dosage, by the DNS method (16). β-Glucosidase activity was determined against *p*-nitrophenyl-β-D-glucoside, and the liberation of *p*-nitrophenol was accompanied by absorption spectroscopy at 410 nm (7). One international unit (U) was defined as the enzymatic activity needed for the release of 1 μmol of glucose equivalents per unit volume and minute of reaction. All measurements were carried out in triplicate and repeated if experimental variation exceeded 10%.

Carbon mass balance. Carbon mass balance closures were calculated as output carbon mass divided by input carbon mass (50): closure (%) = $([\sum C_{out}]/[\sum C_{in}]) \times 100$, where C_{out} is carbon recovery in grams and C_{in} is initial carbon in grams.

The estimation of the carbon mass balance for cellulose degradation and saccharide production on purified cellulose (Avicel) requires information on initial and final carbon contributions, including cellulose concentrations, cell mass concentrations, soluble protein concentrations, concentrations of saccharides, cumulative gaseous carbon dioxide production (total CO_2), and by-prod-

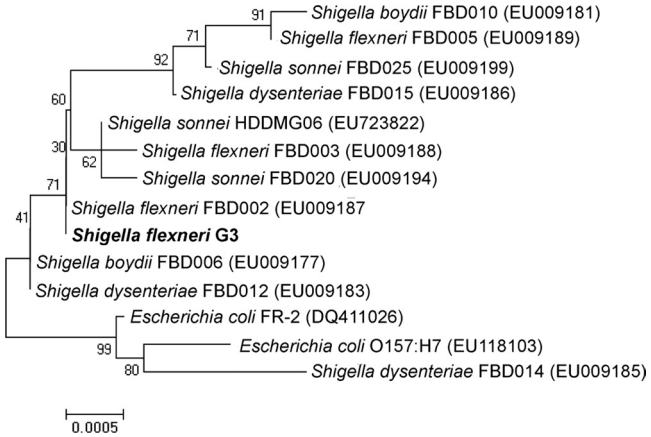

FIG. 1. Phylogenetic tree of strain G3 and other known *Shigella* strains based on 16S rRNA gene sequences constructed by the NJ method. The numbers along branches indicate bootstrap values (1,000 replicates).

ucts (organic acids). All of the parameters were measured immediately after inoculation and thereafter every 5 h until the end of the cultivation.

The carbon contribution from the medium components (primarily yeast extract [see Table S1 in the supplemental material]) was measured with a CHN analyzer (CHNS/O elemental analyzer 2400; Perkin-Elmer, Norwalk, CT). The carbon content of the soluble proteins was estimated to be 50% (wt/wt) of the total protein mass (50). The CO_2 concentration was measured using a gas chromatograph (Agilent Technologies; model 6890N) with argon as the carrier gas (capable of detecting concentrations between 200 and 500 ppm) and equipped with a thermal conductivity detector. CO_2 was analyzed using a stainless steel (3.2 mm by 1.8 mm) column packed with silica gel.

Nucleotide sequence accession number. The 16S rRNA gene nucleotide sequences reported in this paper were submitted to GenBank/EMBL/DDBJ under accession number FJ839361.

RESULTS AND DISCUSSION

Identification and characterization of a cellulolytic saccharifying strain, G3. An enrichment culture was established by incubating rumen fluid in the Avicel mineral salt medium with 0.1% (wt/vol) yeast extract at pH 7.0 and 37°C. Five bacterial strains were isolated, which were able to grow on Avicel as the sole carbon and energy source. All of these isolates showed cellulose degradation and saccharide production activities (data not shown). The strain showing the greatest cellulosic saccharification activity was designated strain G3 and selected for further study. Analysis of the 16S rRNA gene sequence of this strain indicated that it was a member of the genus *Shigella*. Phylogenetic analysis of the 16S rRNA genes showed that G3 clusters with *Shigella boydii*, *Shigella sonnei*, and *Shigella dysenteriae* (Fig. 1), with 99% sequence identity to the 16S rRNA gene of *S. flexneri*.

The physiological properties of *S. flexneri* G3 are summarized in Table 1. Unlike most *Shigella* spp. (3), strain G3 was able to utilize cellulose to produce CO_2 and to hydrolyze various cellulosic materials, such as Avicel, CMC, and filter paper. The majority of *Shigella* spp., including *S. boydii*, *S. flexneri*, and *S. sonnei*, are able to ferment cellobiose, mannitol, etc., but not cellulosic materials. Most strains, except *S. flexneri* 6 and *S. boydii* 13 and 14, ferment sugars without gas production (3). Consistent with other strains of the genus *Shigella*, strain G3 cells are short, rod shaped (0.6 to 1.0 μm by 1.2 to 3.0 μm), and nonmotile (see Fig. S1 in the supplemental material) and exhibit anaerobic growth but do not reduce sulfate or nitrate (Table 1). This strain appears to represent a novel lineage of cellulolytic bacteria.

TABLE 1. Physiological properties of *S. flexneri* G3

Characteristic	Value[a]
Gram staining	−
Morphology	Short rod-shaped, nonspore
Anaerobic growth	+
Motility	−
Sulfate reduction	−
Nitrate reduction	−
Gelatin hydrolysis	+
Oligosaccharide production	+
Metabolic products with cellulose	Cellobiose, glucose, propionate, acetate
Substrate utilization	
Glucose	+
Xylan	−
Galactose	+
Lactose	+
Fructose	+
Maltose	+
Sucrose	−
Cellobiose	−
Xylose	+
Arabinose	−
CMC	+
Salicin	−
Starch	+
Filter paper	+

[a] −, negative; + positive.

Key factors affecting cellulolytic saccharification by strain G3. (i) Yeast extract concentration. Since lignocellulosic material is a relatively nutrient-poor growth substrate, many studies employ rich media for the lignocellulose saccharification process in laboratories. YE has been used extensively in studies of lignocellulose degradation. However, the cost of utilization of YE needs to be considered for any potential commercialization of sugar production from cellulose. Therefore, we first investigated the optimal YE concentration for strain G3 for growth and saccharification. As shown in Table 2, strain G3 was grown in a batch culture at 5.0 g liter of Avicel^{-1}, 35°C, and pH 6.5 with varied concentrations of YE. Our results showed cell growth was improved significantly with the cell generation time sharply decreased from 8.0 h to 4.9 h when the

TABLE 2. Effects of yeast extract on Avicel degradation, saccharification, and cell growth[a]

Amt of YE (g liter^{-1})	Avicel degradation ratio (%)	Amt of oligosaccharides (g liter^{-1})	Cell mass (g liter^{-1})	Cell generation time (h)
0	0	0	0	0
0.5	19.5 ± 1.3	0.37 ± 0.02	0.15 ± 0.02	8.0 ± 0.12
1.0	35.1 ± 2.5	0.75 ± 0.04	0.25 ± 0.02	7.2 ± 0.06
1.5	49.0 ± 1.7	1.25 ± 0.08	0.38 ± 0.04	7.0 ± 0.12
2.0	48.6 ± 1.5	1.18 ± 0.05	0.46 ± 0.03	6.5 ± 0.15
2.5	33.5 ± 2.0	0.71 ± 0.03	0.52 ± 0.05	5.9 ± 0.11
3.0	30.7 ± 2.8	0.65 ± 0.02	0.59 ± 0.03	5.3 ± 0.10
3.5	28.9 ± 1.9	0.60 ± 0.01	0.67 ± 0.02	4.9 ± 0.10

[a] Data are presented as mean ± standard deviation with triplicates after 100 h of incubation at 37°C with 5.0 g liter of Avicel PH-101^{-1}.

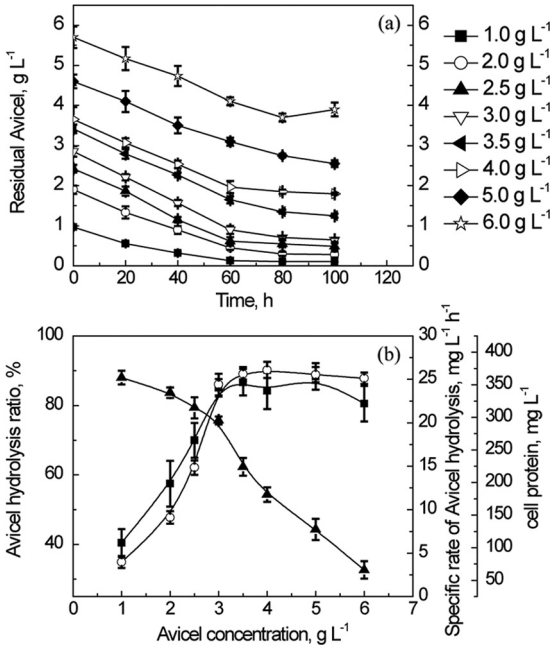

FIG. 2. Avicel hydrolysis by *S. flexneri* strain G3 under different Avicel concentrations over time. (a) Avicel consumption over time. (b) Percentage of hydrolyzed Avicel (▲; left *y* axis), specific rate of Avicel hydrolysis (■; inner right *y* axis), and total protein (○; outer right *y* axis). The data are presented as the mean of triplicate cultures with standard deviations (error bars).

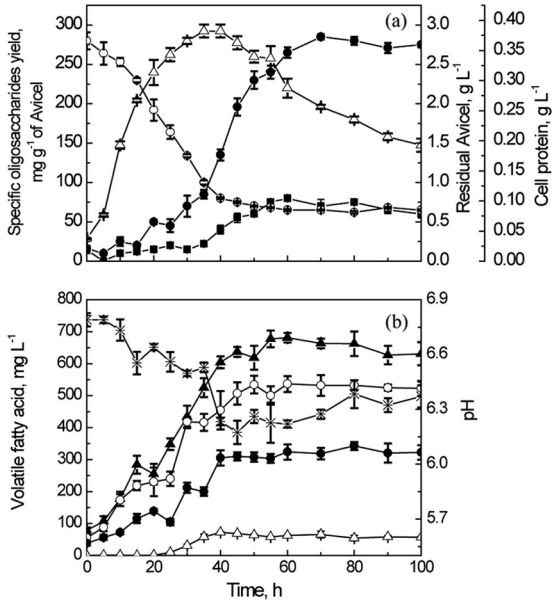

FIG. 3. Kinetics of Avicel degradation over time. (a) Specific oligosaccharide yield (■, glucose; ●, cellobiose; left *y* axis), residual cellulose (○; inner right *y* axis), and cellular protein (△; outer *y* axis). (b) Volatile fatty acids (▲, acetate; ○, propionate; ●, butyrate; △, lactate; left *y* axis) and pH profile (asterisk; right *y* axis) for *S. flexneri* G3 at 5.0 g liter of Avicel PH-101^{-1} within 100 h of batch fermentation of *S. flexneri* G3. The data are presented as the mean of triplicate cultures with standard deviations (error bars).

YE concentration increased from 0.5 to 3.5 g liter^{-1}. However, Avicel degradation was negatively impacted when the YE concentration further increased from 1.5 to 3.5 g liter^{-1}. A similar profile was seen for oligosaccharide production, where oligosaccharides were barely produced at YE concentrations above 2.0 g liter^{-1}. Both the maximum Avicel degradation ratio and the oligosaccharide yield under 5.0 g liter of Avicel^{-1} reached the highest values of 49.0% ± 1.7% and 248 ± 3.7 mg g Avicel^{-1}, respectively, at 1.5 g liter^{-1} YE.

(ii) Temperature and pH. Strain G3 showed cellulose degradation at temperatures ranging from 25°C to 40°C. No cellulose degradation was observed at temperatures below 20°C or above 50°C. The optimal temperature for cell growth was 37°C. The maximum rates of cellulose degradation and oligosaccharide production were 60.0% ± 0.58% and 301.2 ± 2.35 mg g Avicel^{-1} when 5.0 g liter of Avicel^{-1} was used at 40°C, which was in the same range as for *Clostridium cellulovorans* (54).

The optimal pH for cellulose degradation by strain G3 was found to be 6.5, which is consistent with most studies, where cellulose degradation was conducted at pHs ranging from 6.5 to 6.8 (25, 52, 54, 57). Cellulose degradation and oligosaccharide production by strain G3 occurred at a pH range of 5.0 to 8.0. Nearly 55% of the cellulose was hydrolyzed by G3 at pH 6.5 during a 100-hour incubation.

(iii) Avicel concentration. As the maximum saccharification rate was closely linked to cellulose degradation activity (10), the Avicel degradation activities under different substrate concentrations were determined over time. Strain G3 was grown on Avicel with substrate concentrations ranging from 1.0 g liter^{-1} to 6.0 g liter^{-1}. Around 75% Avicel degradation was achieved at lower Avicel concentrations (<3.0 g liter^{-1}), but this percentage dropped to 35% at the highest Avicel concentration (6.0 g liter^{-1}) used in this study (Fig. 2a). The maximum cell protein increased with Avicel concentrations below 3.0 g liter Avicel^{-1}, but it remained constant around 350 mg liter^{-1} above 3.0 g liter Avicel^{-1} (Fig. 2b). The maximum specific rate of cellulose degradation was about 24.1 mg liter^{-1} h^{-1} and was kept constant even at 3.0 g liter Avicel^{-1} or higher.

(iv) Cellulase activity. The total cellulase activity of crude protein samples in pellet and culture supernatants from different substrate concentrations was also investigated (see Fig. S2 in the supplemental material). First, activity reached a peak between 40 and 60 h and then decreased. During hydrolysis with initial Avicel concentrations higher than 3.0 g liter^{-1}, the maximum cellulase activity in the pellet remained quite constant at 0.45 U (see Fig. S2a in the supplemental material). Second, in the supernatant, the cellulase activity appeared after about 24 h and reached higher values ranging from 0.15 to 0.26 U as the initial Avicel concentration increased from 1.0 to 3.0 g liter^{-1} (see Fig. S2b in the supplemental material). However, little cellulase activity (<0.02 U) was detected in the supernatant above 3.0 g liter of Avicel^{-1}, which is consistent with previous studies (10). These results suggest an Avicel concentration of 3.0 g liter^{-1} was optimal for cellulose degradation by G3.

Cellulolytic saccharification by strain G3 under optimum conditions. When G3 was cultivated in the 0.3% Avicel mineral salt medium, cellulose degradation began after an ~5-h lag phase, and about 75% of the cellulose was degraded in the following 60 h (Fig. 3a). This is similar to the rate of cellulose

TABLE 3. Comparison of cellulosic saccharifications from various pure cultures with cellulosic materials as substrates

Microorganism/enzyme preparation	Substrate	Temp (°C)	SY[a]	SPR[d]	Reference
Fungal cultures					
T. reesei Rut30 *T. artoviride* F-1663	SPS[b] (20 g/liter)	30	320	6.60	31
	SPS (20 g/liter)	30	280	5.81	31
Trichoderma aurantiacus	Solka Floc (1 g/liter)	30	300	1.13	18
Aspergillus niger LMA	Cellulose (10 g/liter)	30	500	3.47	1
T. reesei RUT-30 + *A. niger* LMA	Cellulose (10 g/liter)	30	950	6.38	1
Bacterial cultures					
Periconia sp. BC2871	Rice straw (0.025 g/liter)	37	140	104	22
C. cellulolyticum H10	Cellulose (7.8 g/liter)	39	128.2	1.06	9, 17
Bacteroides cellulosolvens + *Clostridium saccharolyticum*	Solka Floc (18 g/liter)	37	200	<1	44
Clostridium acetobutylicum	MC[f] (10 g/liter)	37	12	2.01	62
Clostridium pasteurianum	CMC[e] (10 g/liter)	35	110	ND	37
Enterococcus gallinarum G1	MC[f] (5 g/liter)	37	16	1.1	61
Cellulomonas uda NS1	Rice husk (10 g/liter)	35	282	ND	38
	Rice straw (10 g/liter)	35	206	ND	38
S. flexneri G3	Avicel (3 g/liter)	40	375	6.25	This study
C. thermocellum ATCC 27405	Solka Floc (1 g/liter)	60	370[c]	5.28	29
	Solka Floc (5 g/liter)	60	212[c]	3.02	29
Caldicellulosiruptor kristjanssonii	Avicel (4.0 g/liter)	68	157.5	1.97	52
C. thermocellum JW20	Cellulose (9.7 g/liter)	60	525.9	4.38	15
C. thermocellum JN4	MC (5 g/liter)	60	216	2.16	35
Cellulase preparation from bacterial culture					
Acetivibrio cellulolyticus	Cellulose (20 mg/ml)	40	100	1.10	41
Ruminococcus albus KAM	CMC (32 mg/liter)	45	296	6.08	43

[a] SY, sugar yield (mg g of Avicel^{-1}).
[b] SPS, steam-pretreated spruce.
[c] Value is calculated from the original data.
[d] Specific rate of sugar production (mg g of Avicel^{-1} h^{-1}).
[e] CMC, carboxymethyl cellulose sodium.
[f] MC, microcrystalline cellulose.

degradation observed for *C. thermocellum* at 60°C (33, 36). However, no further cellulose degradation or cell growth was observed after 60 h. This could be due to either the depletion of a particular nutrient from the culture medium (21) or an accumulation of inhibitory intracellular compounds in the medium or in the cell (10, 20). Various types of cellulase enzymes could be inhibited by soluble products (glucose, cellobiose, cellotriose, etc.) via feedback mechanisms (28).

HPLC analysis showed that oligosaccharides were mainly composed of glucose (26%), cellobiose (70%), and trace amounts of cellotriose and cellotetraose (less than 3%) (Fig. 3a; see Fig. S3 in the supplemental material). Specifically, cellobiose was produced with no lag phase and continued to increase until the stationary phase, reaching 282 mg g Avicel^{-1}. This is consistent with the observations of high activities of exoglucanase (0.62 U) and endoglucanase (0.98 U) detected during the hydrolysis-saccharification process. Additionally, most glucose was fermented into the volatile fatty acids, especially at early stages (before 40 h). At late stages, glucose was gradually accumulated and reached a peak yield of 80 mg g Avicel^{-1} at 60 h, which was consistent with the lack of substantial accumulation of fatty acids (Fig. 3b). In order to understand the relationship between carbohydrate utilization and subsequent formation, the kinetics of the utilization of defined carbon sources (1 g liter of glucose or xylose^{-1}) was tested, and the results showed that the yield of volatile fatty acids increased with the decreases in glucose and xylose concentrations (see Fig. S4 in the supplemental material). Cellobiose could not be used by strain G3, and the results are not shown. Cellulose degradation was mainly catalyzed by endo- and exoglucanases and β-glucosidase. However, β-glucosidase, which hydrolyzes cellobiose and other short oligosaccharides to glucose, had a low activity of 0.043 U. This may be another reason why cellobiose was left behind and little glucose was produced after cellulose hydrolysis at an early stage.

In general, oligosaccharide production peaked at 375 mg g Avicel^{-1} during 60 h of cultivation. The specific cellulosic saccharification rate was nearly 6.2 mg g Avicel^{-1} h^{-1}, equivalent to 5.28 mg g Avicel^{-1} h^{-1} from *C. thermocellum* ATCC 27405 at 60°C (Table 3). Furthermore, the primary end products of fermentation included acetate, propionate, and butyrate plus traces of lactate, with acetate-propionate-butyrate molar ratios of 2.3:1.6:1 (Fig. 3b). These results indicate that strain G3 exhibits high hydrolysis activity on Avicel under mesophilic conditions and that oligosaccharide production is even higher than that reported for monocultures of thermophilic *Clostridium* spp. (6, 14, 23, 35).

Carbon balance. The carbon balance of strain G3 was further evaluated based on the utilization of Avicel and the production of cell biomass (total protein), oligosaccharides, liquid end products, and CO_2. The carbon closures ranged from 91.4% to 98.5% for G3. Initially, higher (98.5% ± 0.1%) C balance closures were observed at 5 h. However, the C balance closures decreased slightly to 95.9% ± 0.1% (10 h), 93.0% ± 0.20% (20 h), 93.8% ± 0.45% (30 h), and 93.8% ± 0.55% (40 h). Carbon balance analysis indicated that oligosaccharides are

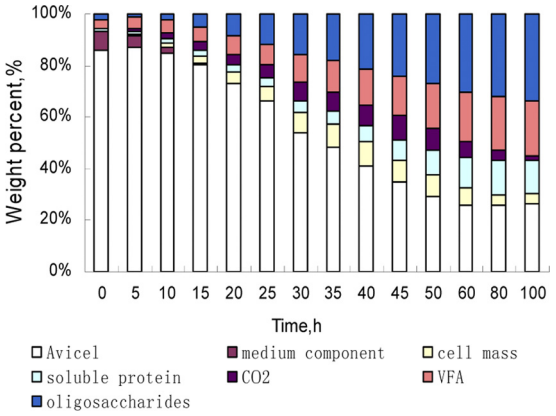

FIG. 4. Dynamics of major extracellular carbon substrates and products, as well as cell biomass, during cellulosic saccharification. The cell biomass was measured as the total protein. The data are presented for each component as the average percentage of all carbon components with 10 replicates.

the dominant components in the later stages (Fig. 4). About 45% of the total carbon was converted to oligosaccharides, and the remainder was used for the production of VFA, CO_2, and soluble protein (Fig. 4). However, this carbon partition was quite different from that in the other studies. Analysis of carbon flow in *C. cellulolyticum* showed that 55 to 70% of the degraded cellulose was converted into end products, such as acetate and lactate, and the rest was converted into biomass, polysaccharides, and proteins (19). Schell et al. (50) also revealed that the majority of carbon from cellulose was converted to CO_2 and cell biomass for *T. reesei* grown on pure cellulose (Solka-Floc), while the proportion of saccharides was near zero. In short, the comparison of carbon balances indicated that *S. flexneri* G3 produces a higher concentration of saccharides than other known cellulosic microorganisms, especially under mesophilic conditions (Table 3).

Concluding remarks. A novel mesophilic bacterium, *S. flexneri* G3, capable of producing oligosaccharides efficiently from Avicel, was isolated and characterized. This is, to the best of our knowledge, the first mesophilic cellulolytic bacterium with the highest reported saccharification capability from cellulose. This is also the first report of a strain in the genus *Shigella* capable of cellulosic saccharification. This strain could be useful for various types of biotechnologies in hydrogen production, waste treatment, and other energy product production from cellulosic materials.

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