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Periplasmic *Cytophaga hutchinsonii* Endoglucanases Are Required for Use of Crystalline Cellulose as the Sole Source of Carbon and Energy

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ABSTRACT

The soil bacterium *Cytophaga hutchinsonii* actively digests crystalline cellulose by a poorly understood mechanism. Genome analyses identified nine genes predicted to encode endoglucanases with roles in this process. No predicted cellobiohydrolases, which are usually involved in the utilization of crystalline cellulose, were identified. Chromosomal deletions were performed in eight of the endoglucanase-encoding genes: *cel5A*, *cel5B*, *cel5C*, *cel9A*, *cel9B*, *cel9C*, *cel9E*, and *cel9F*. Each mutant retained the ability to digest crystalline cellulose, although the deletion of *cel9C* caused a modest decrease in cellulose utilization. Strains with multiple deletions were constructed to identify the critical cellulases. Cells of a mutant lacking both *cel5B* and *cel9C* were completely deficient in growth on cellulose. Cell fractionation and biochemical analyses indicate that Cel5B and Cel9C are periplasmic nonprocessive endoglucanases. The requirement of periplasmic endoglucanases for cellulose utilization suggests that cellodextrins are transported across the outer membrane during this process. Bioinformatic analyses predict that Cel5A, Cel9A, Cel9B, Cel9D, and Cel9E are secreted across the outer membrane by the type IX secretion system, which has been linked to cellulose utilization. These secreted endoglucanases may perform the initial digestion within amorphous regions on the cellulose fibers, releasing oligomers that are transported into the periplasm for further digestion by Cel5B and Cel9C. The results suggest that both cell surface and periplasmic endoglucanases are required for the growth of *C. hutchinsonii* on cellulose and that novel cell surface proteins may solubilize and transport cellodextrins across the outer membrane.

IMPORTANCE

The bacterium *Cytophaga hutchinsonii* digests crystalline cellulose by an unknown mechanism. It lacks processive cellobiohydrolases that are often involved in cellulose digestion. Critical cellulolytic enzymes were identified by genetic analyses. Intracellular (periplasmic) nonprocessive endoglucanases performed an important role in cellulose utilization. The results suggest a model involving partial digestion at the cell surface, solubilization and uptake of cellodextrins across the outer membrane by an unknown mechanism, and further digestion within the periplasm. The ability to sequester cellodextrins and digest them intracellularly may limit losses of soluble cellobiose to other organisms. *C. hutchinsonii* uses an unusual approach to digest cellulose and is a potential source of novel proteins to increase the efficiency of conversion of cellulose into soluble sugars and biofuels.

Cytophaga hutchinsonii is a common cellulolytic soil bacterium that belongs to the phylum *Bacteroidetes* (1). Cells of *C. hutchinsonii* digest crystalline cellulose and grow with filter paper as the sole source of carbon and energy. *C. hutchinsonii* specializes in cellulose digestion and is known to grow only on cellulose or cellulose digestion products (1, 2). Direct contact of cells with their insoluble substrate is required for efficient digestion (1). Although *C. hutchinsonii* and closely related cellulolytic bacteria have been known for almost 100 years (3–6), the mechanism by which they digest cellulose is not understood.

Other cellulolytic bacteria that have been well studied either secrete soluble cellulolytic enzymes or produce multiprotein cell surface or extracellular cellulosomes (7–9). Cellulosomes contain a collection of enzymes and cellulose-binding proteins that participate in cellulose digestion. The proteins that make up the cellulosomes contain dockerin and cohesin domains that facilitate the protein-protein interactions needed to form the final structures. In both the soluble cellulase and the cellulosome strategies, endo-acting β-1,4-endoglucanases and exo-acting cellobiohydrolases typically function synergistically to digest cellulose. The endoglucanases attack noncrystalline amorphous regions of the cellulose fibers, generating sites of attack for the cellobiohydrolases that processively release the disaccharide cellobiose and disrupt the crystalline structure of cellulose. Most endoglucanases are nonprocessive, but a few processive endoglucanases that may contribute to the disruption of crystalline cellulose are also known (10, 11). Some of these contain family 3 carbohydrate-binding modules (CBM3) that are thought to maintain contact with the cellulose strand and allow the endoglucanase to digest processively (11). Lytic polysaccharide monooxygenases (LPMOs) that cleave glycosidic bonds using an oxidative mechanism have also been identified in many aerobic cellulolytic bacteria (12, 13). A

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TABLE 1 Predicted *C. hutchinsonii* endoglucanases involved in cellulose digestion

Protein	Locus	Molecular mass (kDa)[a]	Predicted localization[b]	Modular structure[c]
Cel5A	CHU_1107	135.2	Extracellular/cell surface	SP-GH5-X1-PKD-PKD-FN3-CTD
Cel5B	CHU_2103	38.8	Periplasmic	SP-GH5
Cel5C	CHU_1727	67.9	Periplasmic	SP-GH5
Cel9A	CHU_1336	105.3	Extracellular/cell surface	SP-GH9-PKD-PKD-PKD-CTD
Cel9B	CHU_1335	207.3	Extracellular/cell surface	SP-GH9-PKD-PKD-PKD-BIG-BIG-BIG-BIG-BIG-BIG-BIG-BIG-BIG-BIG-CTD
Cel9C	CHU_1280	65.1	Periplasmic	SP-CelD_N-GH9
Cel9D	CHU_1655	92.6	Extracellular/cell surface	SP-CelD_N-GH9-CTD
Cel9E	CHU_0778	81.7	Extracellular/cell surface	SP-CelD_N-GH9-CTD
Cel9F	CHU_2235	64.2	Cytoplasmic membrane/periplasmic[d]	GH9[d]

[a] Molecular mass of primary product of translation, including predicted signal peptide.
[b] Localization predicted based on predicted cleavable N-terminal signal peptide by SignalP version 4.1 (61) for transit of cytoplasmic membrane and of type IX secretion system C-terminal-targeting domain (CTD) belonging to family TIGR04183 or TIGR04131.
[c] Modular structure is indicated by the following abbreviations: SP, type I signal peptide; BIG, bacterial immunoglobulin-like domain group 2 (Pfam no. PF02368); CelD_N, N-terminal immunoglobulin-like domain of cellulase (Pfam no. PF02927); CTD, type IX secretion system C-terminal sorting domain (TIGR04183); FN3, fibronectin type 3 domain (Pfam no. PF00041); GH, glycoside hydrolase (number indicates family), as assigned by CAZY; PKD, polycystic kidney disease protein PKD1 (Pfam no. PF00801); and X1, conserved domain of unknown function (1).
[d] Cel9F was not predicted to have a cleavable N-terminal signal peptide by SignalP. However, it has an obvious hydrophobic N-terminal sequence that may target it for export across the cytoplasmic membrane by the Sec protein export system. If this region is cleaved, Cel9F should localize to the periplasm, whereas if it is not cleaved, Cel9F should localize to the cytoplasmic membrane.

combination of hydrolytic and oxidative mechanisms is thought to be used by these organisms to digest cellulose (13).

C. hutchinsonii has many unique aspects to its cellulolytic machinery. *C. hutchinsonii* does not use either free cellulase or cellulosome strategies. Its known cellulolytic enzymes are cell associated, but they lack dockerin and cohesin domain-containing proteins that are characteristic of cellulosomes (1). Analysis of the *C. hutchinsonii* genome revealed nine predicted β-1,4-endoglucanases thought to be involved in cellulose utilization (Table 1) but no predicted cellobiohydrolases (1). Its endoglucanases lack predicted cellulose-binding modules (1), suggesting that they may be nonprocessive. Although *C. hutchinsonii* is an aerobic cellulose-digesting bacterium, it lacks genes encoding homologs of known LPMOs. The apparent absence of recognizable cellobiohydrolases, processive endoglucanases, and LPMOs raises the question of how *C. hutchinsonii* solubilizes crystalline cellulose. Some of the unusual traits of *C. hutchinsonii* are shared with the distantly related anaerobic rumen bacterium *Fibrobacter succinogenes*. Like *C. hutchinsonii*, *F. succinogenes* digests crystalline cellulose without the assistance of obvious cellobiohydrolases and LPMOs. Further studies of the *F. succinogenes* cellulolytic system have been hampered by the lack of tools to genetically manipulate the organism. A speculative model of *C. hutchinsonii* and *F. succinogenes* cellulose utilization involves partial digestion by extracellular endoglucanases, solubilization and uptake of cellodextrins into the periplasm by an unknown mechanism, and digestion of these by periplasmic endoglucanases and β-glucosidases (14, 15).

Genetic techniques recently developed for *C. hutchinsonii* allow questions related to its unusual cellulose utilization machinery to be examined (16–22). Three of the nine predicted endoglucanase-encoding genes, *cel5A* (CHU_1107), *cel5B* (CHU_2103), and *cel9B* (CHU_1335), have been disrupted by insertion mutagenesis, but these single-gene mutations did not result in obvious defects in cellulose utilization (22, 23).

In this study, a series of gene deletions were constructed to test the involvement of eight predicted endoglucanase-encoding genes in cellulose utilization. The deletion of single endoglucanase-encoding genes had little effect on cellulose utilization, but strains lacking both *cel5B* and *cel9C* (CHU_1280) failed to utilize cellulose as the sole source of carbon and energy. Cel9C and Cel5B appear to be nonprocessive periplasmic endoglucanases that perform essential functions in *C. hutchinsonii* cellulose utilization. The requirement of periplasmic endoglucanases for cellulose utilization suggests that cellodextrins are transported across the outer membrane by an unknown mechanism. The apparent ability to sequester cellodextrins and digest them intracellularly may be an advantage that limits losses of soluble cellobiose and glucose to other microorganisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The streptomycin-resistant *C. hutchinsonii* strain CH428, which was derived from the *C. hutchinsonii* type strain ATCC 33406 to facilitate the construction of gene deletions, was the wild-type strain used in this study (21). *C. hutchinsonii* strains were grown in PY10 liquid medium (10.0 g/liter peptone, 0.5 g/liter yeast extract, 4.0 g/liter glucose [pH 7.3]) (22) at 25°C. PY10 agar was identical except for the addition of agar (6 g/liter, unless indicated otherwise). Cells from freezer stocks were propagated on PY10 agar at 25°C for 7 days. Actively growing cells from these plates were used to inoculate the growth media for all experiments. To analyze the digestion of filter paper and growth on Avicel PH-101, cells were grown on Stanier medium (1.0 g/liter KNO_3, 1.0 g/liter K_2HPO_4, 0.2 g/liter $MgSO_4 \cdot 7H_2O$, 0.1 g/liter $CaCl_2 \cdot 2H_2O$, 0.02 g/liter $FeCl_3 \cdot 6H_2O$ [pH 7.3]) (24), with cellulose as the only available source of carbon and energy at 25°C. *Escherichia coli* strains were grown at 37°C in Luria-Bertani medium (25). Antibiotics were used at the following concentrations when needed: ampicillin, 100 μg/ml; chloramphenicol, 20 μg/ml; erythromycin, 60 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 100 μg/ml. The strains and plasmids used in this study are listed in Table S1 in the supplemental material. The primers used in this study are listed in Table S2 in the supplemental material.

Construction and complementation of mutants. In-frame chromosomal deletions were constructed for eight of the nine predicted endoglucanase-encoding genes of *C. hutchinsonii* by using the *rpsL*-containing suicide vectors pYT282 (see Fig. S1 in the supplemental material) and pYT160, essentially as previously described (21) (see Supplemental Methods in the supplemental material for details). For complementation ex-

periments, *C. hutchinsonii cel5B* and *cel9C* were amplified from wild-type cells, cloned into pYT162, and introduced into *C. hutchinsonii* mutants by conjugation, as previously described (21) (see Supplemental Methods in the supplemental material for details).

Digestion of filter paper, regenerated amorphous cellulose (RAC), and Avicel cellulose by *C. hutchinsonii* cells. Wild-type and mutant *C. hutchinsonii* strains were grown at 25°C for 2 days on PY10 with 15 g/liter agar, at which time they were scraped off the plates, suspended in 1 ml of Stanier medium, and pelleted by centrifugation at 4,200 × *g* for 3 min to remove residual glucose. Cells were suspended in Stanier medium to a concentration (optical density at 600 nm [OD_{600}]) of 1.0. Cells (3 μl) were spotted on 30-mm-diameter Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, United Kingdom) or on 1.5 ml of 1% RAC, prepared as previously described (2, 26), that was overlaid on Stanier agar in 55-mm-diameter petri dishes. For some experiments, digestion of cellulose was examined on Stanier agar containing 0.1% glucose. Wild-type and mutant cells were also spotted on Stanier agar containing 0.1% glucose or 0.1% cellobiose without cellulose to determine if there were general growth defects. Cells were incubated at 25°C, and growth and cellulose digestion were documented using a Kodak DC290 camera (Eastman Kodak Co., Rochester, NY). Growth of cells in broth culture on Avicel PH-101 cellulose was determined as previously described (27) (see Supplemental Methods in the supplemental material for details).

Assay of bacterial adhesion to cellulose. Bacterial adhesion to cellulose was measured, as previously described (27), by mixing cells with Avicel PH-101, sedimenting the cellulose particles and attached bacteria by centrifugation at 100 × *g* for 1 min, and measuring the optical density of the supernatants (see Supplemental Methods in the supplemental material for details).

Expression and analysis of recombinant Cel5B and Cel9C. *C. hutchinsonii* Cel5B and Cel9C were expressed in *E. coli* and purified as described in the supplemental material. Polyclonal antibodies against recombinant Cel9C or Cel5B were produced using recombinant proteins by the Biomatik Corporation (Cambridge, Ontario, Canada). The processivity of Cel5B and Cel9C was determined, essentially as previously described (28), by incubating with crystalline bacterial cellulose (BC) as the substrate and measuring the distribution of reducing sugars between soluble and insoluble fractions (see Supplemental Methods in the supplemental material for details). *Thermobifida fusca* Cel6B (29) and Cel9B (30) were used as controls for processive and nonprocessive enzymes, respectively. The crystal structure of *C. hutchinsonii* Cel5B was determined as described in the supplemental material.

Localization of Cel5B and Cel9C in *C. hutchinsonii* cells. Strains were grown in Stanier medium with 0.1% glucose as the carbon source at 25°C with shaking. Cultures were harvested at the beginning of stationary phase by centrifugation at 3,700 × *g* for 10 min to obtain cell pellets (intact cells) and supernatant fractions (spent medium). Cell pellets were washed once with Stanier medium and suspended in Stanier medium. Spent-medium samples were centrifuged at 352,900 × *g* for 15 min to remove any remaining cells. Proteins from 400 μl of spent medium were precipitated with 20% trichloroacetic acid and retained as the soluble secreted protein (spent-medium) fraction. Cells from 400 μl of original cultures were pelleted and retained as the cell fraction. Halt protease inhibitor (Thermo Fisher Scientific, Waltham, MA) was added to cells in Stanier medium (OD_{600}, 2.0), and cells were lysed with a French pressure cell. Unbroken cells and debris were removed by centrifugation twice for 10 min at 16,800 × *g*. Seven hundred microliters of cell extract was centrifuged for 30 min at 352,900 × *g*. The supernatant was retained as the soluble (cytoplasm and periplasm) fraction, and the pellet was washed once and suspended with 700 μl of Stanier medium and retained as the membrane fraction.

Periplasm preparation was performed using the Epicentre PeriPreps Periplasting kit (Epicentre Technologies, Madison, WI). Cells from 1 ml of an exponentially growing culture (OD_{600}, 1.0) in Stanier medium were harvested by centrifugation at 10,000 × *g* for 5 min. The cells were suspended in 100 μl of buffer containing 20 mM Tris-HCl (pH 8.0), 20% sucrose, 1 mM EDTA, and 40 U/μl of lysozyme, followed by incubation for 30 min at room temperature (20°C). One hundred microliters of ice-cold double-distilled water was added to the cells, mixed by inversion, incubated for 5 min on ice, and centrifuged for 15 min at 5,000 × *g*. The supernatant was retained as the periplasm-enriched fraction, and the pellet was suspended with 200 μl of Stanier medium and retained as the cytoplasm/membrane-enriched fraction. Proteins were separated by SDS-PAGE (10% acrylamide running gel), and Western blot analyses were performed as previously described (31).

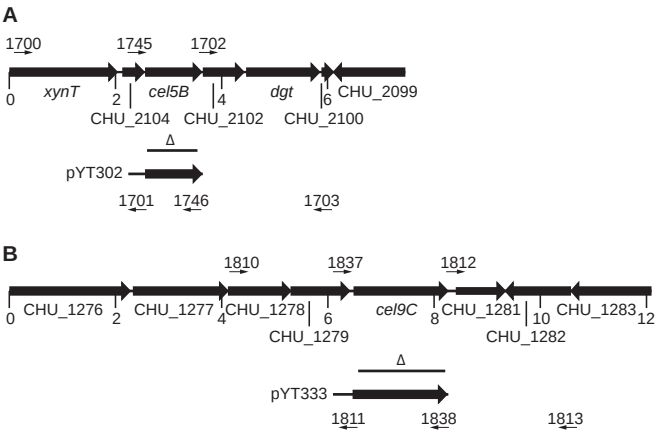

FIG 1 Maps of the regions containing *cel5B* (A) and *cel9C* (B) and associated deletions. Numbers below the maps refer to kilobase pairs of sequence. Binding sites for primers used in PCRs to generate deletion constructs or to complement the final deletion mutations are shown above and below the maps, with the blunt ends indicating the actual binding sites. The horizontal lines beneath the maps marked with open triangles denote regions deleted from the chromosome in the mutants. The regions of DNA carried by complementation plasmids pYT302 and pYT333 are indicated beneath the maps.

Proteinase K treatment of cells was used to determine if Cel5B and Cel9C were exposed on the cell surface. In brief, proteinase K was added to cells of *C. hutchinsonii* to digest exposed proteins, and Western blot analyses were performed to detect Cel5B and Cel9C (see Supplemental Methods in the supplemental material for details).

Accession number(s). Coordinates and structure factors for the Cel5B model have been deposited in the Protein Data Bank (www.rcsb.org) with the accession no. 5IHS.

RESULTS

Mutants lacking individual predicted endoglucanase-encoding genes retained the ability to digest cellulose. Nine genes predicted to encode endoglucanases involved in cellulose digestion (Table 1) were identified by analysis of the *C. hutchinsonii* genome (1). The recent development of gene deletion techniques for *C. hutchinsonii* (21) allowed us to determine if individual predicted endoglucanase-encoding genes were required for cellulose utilization. Mutants lacking individual genes (*cel5A*, *cel5B*, *cel5C*, *cel9C*, *cel9E*, and *cel9F*) and a mutant lacking the adjacent genes *cel9A* and *cel9B* were constructed (Fig. 1; see also Fig. S2 in the supplemental material) and analyzed for the ability to digest filter paper cellulose, Avicel, and regenerated amorphous cellulose (RAC) (Fig. 2). RAC, derived from Avicel by phosphoric acid dissolution and regeneration, is more hydrated than crystalline cellulose and has fewer hydrogen bonds between cellulose chains (11, 26). Each mutant except the Δ*cel9C* mutant digested filter paper and Avicel as well as wild-type cells and grew with cellulose as the sole source of carbon and energy. Cells of the Δ*cel9C* mutant also digested and

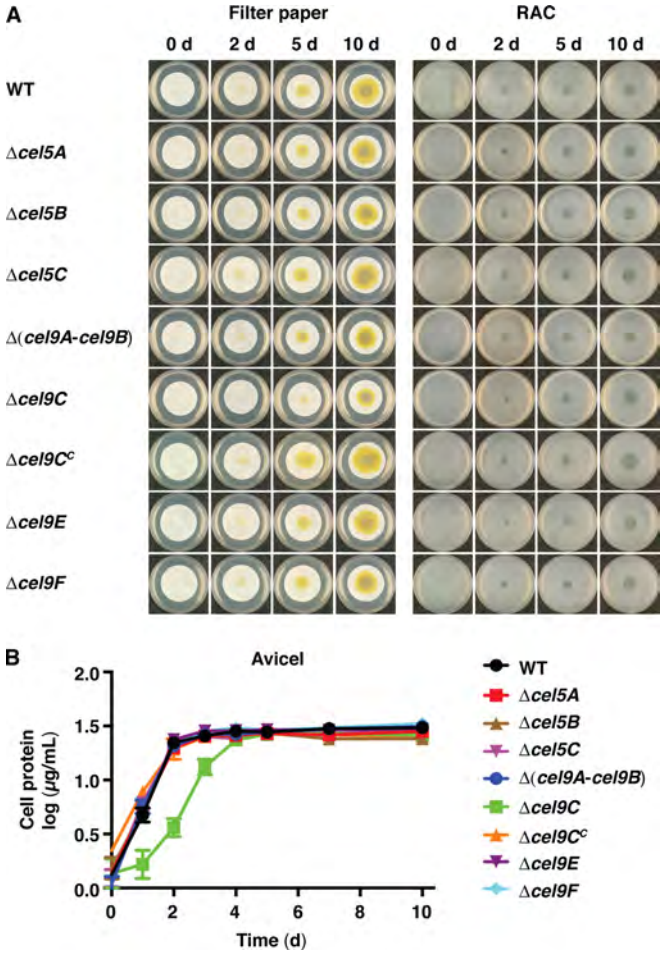

FIG 2 Effect of single deletions of predicted endoglucanase-encoding genes on ability to utilize cellulose as sole source of carbon and energy. (A) Equal amounts of cells (3 μl; OD_{600}, 1.0) of wild-type (WT) and mutant strains were spotted on 30-mm-diameter Whatman filter paper on Stanier agar or on RAC that was overlaid on Stanier agar. Cells were incubated at 25°C. Clear zones indicate digestion of cellulose. Δ*cel9C*^{C} indicates complementation of the Δ*cel9C* mutant with *cel9C* on pYT333. Growth on filter paper and RAC was performed in duplicate (with the same results), and one set of data is shown. (B) Growth of mutants on Avicel cellulose. Cells (0.1 ml; OD_{600}, 1.0) were inoculated into 50 ml of Stanier medium supplemented with 0.1% Avicel PH-101 in 250-ml flasks and incubated with shaking at 25°C. Growth is presented as log (micrograms of cell protein per milliliter). Growth curves were performed in triplicate, and the error bars indicate standard deviations. d, days.

grew on filter paper and Avicel, but they exhibited a modest growth defect compared to wild-type cells, although the final biomass was similar (Fig. 2). The Δ*cel9C* mutant complemented with pYT333 (carrying wild-type *cel9C*) digested and grew on crystalline cellulose as well as the wild type. The Δ*cel9C* mutant and all of the other single-deletion mutants (Δ*cel5A*, Δ*cel5B*, Δ*cel5C*, Δ*cel9AB*, Δ*cel9E*, and Δ*cel9F*) grew as well as the wild type on RAC (Fig. 2A). The results indicate that none of these eight genes are essential for cellulose digestion. Multiple attempts were made to delete the ninth predicted endoglucanase-encoding gene, *cel9D*, without success. This failure does not imply that *cel9D* is essential for viability, because we did not even obtain strains with the deletion constructs pYT278 and pYT336 (see Table S1 in the supplemental material) integrated into the chromosome, which should not have disrupted the expression of *cel9D* or of any other genes.

Mutants lacking *cel5B*, *cel9A*, and *cel9B* were partially defective in cellulose utilization. The ability of the single-deletion mutants described above to digest cellulose suggested the possibility that some of the endoglucanases may exhibit a redundancy of function. The gene deletion approach was used iteratively to construct strains lacking multiple predicted endoglucanase-encoding genes. The Δ*cel5B* Δ*cel9AB* mutant utilized RAC well but exhibited a minor growth defect on Avicel compared with the wild type (see Fig. S3 in the supplemental material). This defect was partially complemented by pYT302, which carries *cel5B*. Cells of the Δ*cel5B* Δ*cel9A* and Δ*cel5B* Δ*cel9B* double-mutant strains grew as well as wild-type cells on all types of cellulose that were tested (Fig. S3). The growth defect on Avicel cellulose was only observed when *cel5B*, *cel9A*, and *cel9B* were all missing. The deletion of four additional endoglucanase-encoding genes (*cel5A*, *cel5C*, *cel9E*, and *cel9F*) from the Δ*cel5B* Δ*cel9AB* mutant resulted in a strain lacking seven of the nine predicted endoglucanase-encoding genes. This mutant exhibited the same minor growth defect on crystalline cellulose as the Δ*cel5B* Δ*cel9AB* mutant (Fig. S3B).

Mutants lacking both *cel5B* and *cel9C* failed to digest cellulose. *cel9C* was deleted in CH509, which lacks five endoglucanase-encoding genes: *cel5A*, *cel5B*, *cel9A*, *cel9B*, and *cel9E*. The resulting mutant failed to grow on filter paper cellulose (data not shown). To identify the critical endoglucanases, a series of mutants lacking *cel9C* and other endoglucanase-encoding genes were constructed. The Δ*cel5B* Δ*cel9C* mutant CH542 failed to grow on filter paper, Avicel, and RAC (Fig. 3). Plasmids pYT302 carrying *cel5B* and pYT333 carrying *cel9C* restored cellulose digestion ability to the mutant. The Δ*cel5B* Δ*cel9C* mutant grew as well as the wild type when glucose was supplied as a carbon and energy source, demonstrating that it did not have a general growth defect (see Fig. S4 in the supplemental material). The mutant CH625, lacking six endoglucanase-encoding genes (*cel5A*, *cel5C*, *cel9A*, *cel9B*, *cel9E*, and *cel9F*), utilized cellulose and grew as well as the wild type (see Fig. S5 in the supplemental material), indicating that these predicted endoglucanase-encoding genes are not required for cellulose utilization, although they may have semiredundant roles in this process. The deletion of either *cel5B* or *cel9C* in CH625 resulted in mutants that were only partially defective in cellulose utilization (Fig. S5). Similarly, complementation of the cellulose-nonutilizing mutant CH585, which lacks eight endoglucanase-encoding genes (*cel5A*, *cel5B*, *cel5C*, *cel9A*, *cel9B*, *cel9C*, *cel9E*, and *cel9F*), with either wild-type *cel5B* or *cel9C* restored the ability to digest cellulose (Fig. 3). The results demonstrate the importance of Cel5B and Cel9C in cellulose digestion and suggest partial redundancy between these endoglucanases. Mutants lacking both *cel5B* and *cel9C* grew as well as the wild type when cellobiose was supplied as a carbon and energy source, indicating that they are specifically required for cellulose utilization (see Fig. S4 in the supplemental material).

Wild-type *C. hutchinsonii* binds tightly to cellulose, and this is thought to be required for its efficient digestion (1). Each of the single- and multiple-deletion mutants, including the strain lacking eight of the nine predicted endoglucanase-encoding genes, retained the ability to bind cellulose. The percentage of wild-type cells, and of CH542 (Δ*cel5B* Δ*cel9C*) and CH585 (Δ*cel5A* Δ*cel5B* Δ*cel5C* Δ*cel9AB* Δ*cel9C* Δ*cel9E* Δ*cel9F*) mutant cells that attached to Avicel during 1 h of incubation were 85.9% (standard devia-

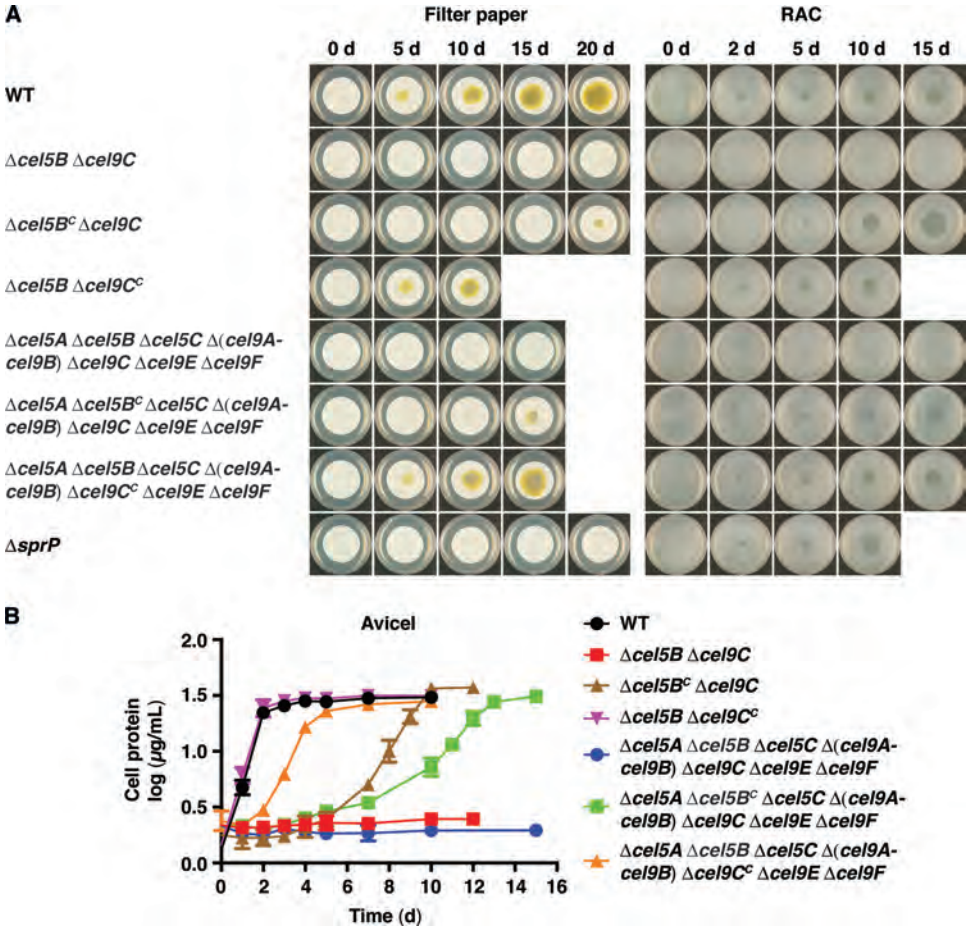

FIG 3 Effect of deletion of *cel5B* and *cel9C* on ability to utilize cellulose as sole source of carbon and energy. (A) Equal amounts of cells (3 μl; OD_{600}, 1.0) of wild-type (WT) and mutant strains were spotted on Whatman filter paper or on RAC that was overlaid on Stanier agar. Cells were incubated at 25°C. Clear zones indicate digestion of cellulose. Δ*cel5B*^{C} and Δ*cel9C*^{C} indicate complementation of the *cel5B* and *cel9C* deletion mutations with *cel5B* and *cel9C* on plasmids pYT302 and pYT333, respectively. Growth on filter paper and RAC was performed in duplicate (with the same results), and one set of data is shown. (B) Growth of mutants on Avicel cellulose. Cells (0.1 ml; OD_{600}, 1.0) were inoculated into 50 ml of Stanier medium supplemented with 0.1% Avicel PH-101 in 250-ml flasks and incubated with shaking at 25°C. Growth is presented as log (micrograms of cell protein per milliliter). Growth curves were performed in triplicate, and the error bars indicate standard deviations.

tion, 4.9%), 93.4% (standard deviation, 2.9%), and 91.4% (standard deviation, 1.0%), respectively, indicating that the cellulose utilization defects of the mutants did not result from decreased binding to cellulose. Analysis of the *C. hutchinsonii* genome failed to detect obvious cellulose-binding domains on any proteins (1), but recent experiments identified several novel membrane proteins that bind to cellulose (17, 32). The domains involved in binding cellulose and their exact roles in cellulose digestion remain to be determined.

T9SS and cellulose utilization. *C. hutchinsonii*, like many other members of the phylum *Bacteroidetes*, secretes numerous proteins across its outer membrane using the type IX secretion system (T9SS) (21, 33). Proteins targeted for secretion by T9SSs have conserved carboxy-terminal domains (CTDs) belonging to TIGRFAM protein domain families TIGR04183 and TIGR04131, which are involved in this process (34). The addition of these CTDs to foreign proteins results in their secretion (35–37). Previous work showed that the deletion of *C. hutchinsonii sprP*, which encodes an accessory component of the T9SS, resulted in a defect in protein secretion and inability to grow on filter paper cellulose (21). The endoglucanases Cel5A, Cel9A, Cel9B, Cel9D, and Cel9E have T9SS CTDs (Table 1). As demonstrated above, Cel5A, Cel9A, Cel9B, and Cel9E are not essential for cellulose utilization, but Cel9D may be required for this process. Other *C. hutchinsonii* proteins besides endoglucanases also require the T9SS for delivery across the outer membrane (21, 33), and the lack of secretion of some of these might contribute to the cellulose utilization defect of the *sprP* mutant. The Δ*sprP* mutant does not grow on crystalline cellulose, such as filter paper and Avicel (21), but it digested and grew well on RAC as the sole source of carbon and energy (Fig. 3). This suggests that proteins that require SprP for secretion are needed for the utilization of crystalline cellulose but not for the utilization of amorphous cellulose. SprP is thought to be required for the secretion of only a subset of proteins targeted to the T9SS (21), and it is likely that some proteins involved in cellulose utilization are secreted by the Δ*sprP* mutant, thus allowing growth on RAC. Cel5B and Cel9C, which, as shown above, are required for the utilization of crystalline and amorphous cellulose as the sole sources of carbon and energy, do not have predicted T9SS CTDs (Table 1) and thus are probably not secreted by the T9SS. While

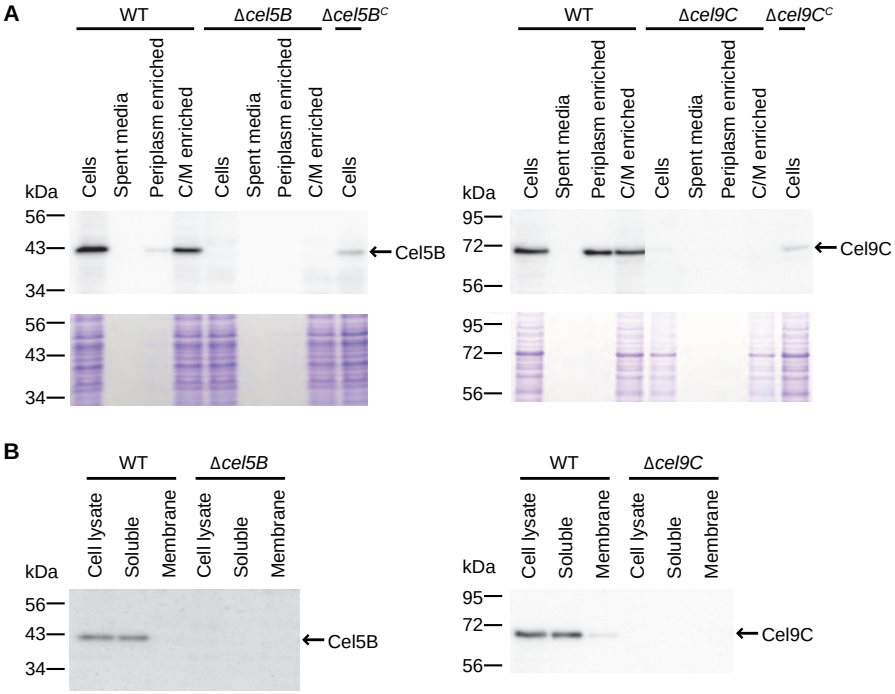

FIG 4 Localization of Cel5B and Cel9C by Western blot immunodetection. (A) Immunodetection of Cel5B and Cel9C in whole cells, spent media, and in periplasm-enriched and cytoplasm/membrane (C/M)-enriched cell fractions. Shown below the Western blots are Coomassie brilliant blue-stained gels with identical samples. Cells of wild type (WT), Δ*cel5B* mutant, Δ*cel5B* mutant complemented with pYT302, Δ*cel9C* mutant, and Δ*cel9C* mutant complemented with pYT333 were examined. (B) Immunodetection of Cel5B and Cel9C in whole-cell lysates and in soluble and membrane fractions of cell lysates. Cells were lysed with a French pressure cell, and membranes were pelleted by ultracentrifugation. Cells of wild type, Δ*cel5B* mutant, and Δ*cel9C* mutant strains were examined.

the cells of the Δ*cel5B* Δ*cel9C* mutant failed to grow on RAC (Fig. 3), cells of the Δ*cel5B* Δ*sprP* and Δ*cel9C* Δ*sprP* mutants digested and grew on RAC as well as the wild-type cells (see Fig. S6 in the supplemental material).

Cel5B and Cel9C appear to be periplasmic endoglucanases. Cel5B and Cel9C are predicted to have N-terminal signal peptides for export across the cytoplasmic membrane by the secretory (Sec) system, but they lack the conserved CTDs that target proteins to the T9SS for secretion across the outer membrane. A previous study indicated that Cel9C was 571 amino acids long and lacked a signal peptide (38), but manual annotation identified a more likely start codon that resulted in a translated protein of 589 amino acids, with an obvious hydrophobic signal peptide at the amino terminus (see Fig. S7 in the supplemental material). We examined the localization of Cel5B and Cel9C by cell fractionation and Western blot analyses. Neither protein was secreted into the culture fluid, since they were not detected in the cell-free spent-culture medium (Fig. 4A). Total membranes and soluble fractions (containing periplasmic and cytoplasmic proteins) were separated by ultracentrifugation. Cel5B and Cel9C were detected only in the soluble fractions of the cell extracts (Fig. 4B). Cel5B and Cel9C are soluble cell-associated proteins that are predicted to have cleavable type I N-terminal signal peptides (Table 1). This suggests that they reside in the periplasm. Treatment of cells with osmotic shock (Epicenter PeriPreps Periplasting kit), a procedure used to isolate periplasmic contents, resulted in a protein fraction that was enriched for Cel9C and, to a lesser extent, Cel5B (Fig. 4A).

As an independent approach to determine the localization of Cel5B and Cel9C, cells were treated with proteinase K to digest cell surface proteins while leaving proteins that reside within the cell undigested. Proteinase K failed to digest Cel5B and Cel9C unless the cells had been disrupted with a French press, indicating that the proteins are not on the cell surface (Fig. 5). Taken together, the experimental results and bioinformatic analyses indicate that Cel5B and Cel9C are not secreted across the outer membrane but instead reside in the periplasm.

Strains lacking Cel5B and Cel9C retain some ability to digest cellulose when provided with glucose. The results described above indicate that Cel5B and Cel9C are required for utilization of cellulose as the sole source of carbon and energy. However, two lines of reasoning suggest that some cellulose digestion should occur in Δ*cel5B* Δ*cel9C* double mutants. First, although Cel5B and Cel9C appear to be periplasmic, some other endoglucanases are predicted to be present on the cell surface (1, 21). These proteins should attack cellulose even in the absence of Cel5B and Cel9C. Second, the requirement of periplasmic Cel5B and Cel9C for growth on cellulose suggests that active endoglucanases must be present outside the cell to release cellodextrins that can be transported into the periplasm. Most of the remaining endoglucanases (Cel5A, Cel5C, Cel9A, Cel9B, Cel9E, and Cel9F) are not essential for cellulose utilization (see Fig. S5 in the supplemental material), but Cel9D, which is similar in sequence to Cel9C but has a T9SS CTD (see Fig. S7 in the supplemental material), should be present in each mutant and might be involved in partial digestion of extracellular cellulose. To examine this possibility, we grew cells of the Δ*cel5B* Δ*cel9C* mutant on cellulose in the presence of glucose. The results (Fig. 6) indicate that although the Δ*cel5B* Δ*cel9C* mutant did not grow on cellulose as a sole source of carbon and

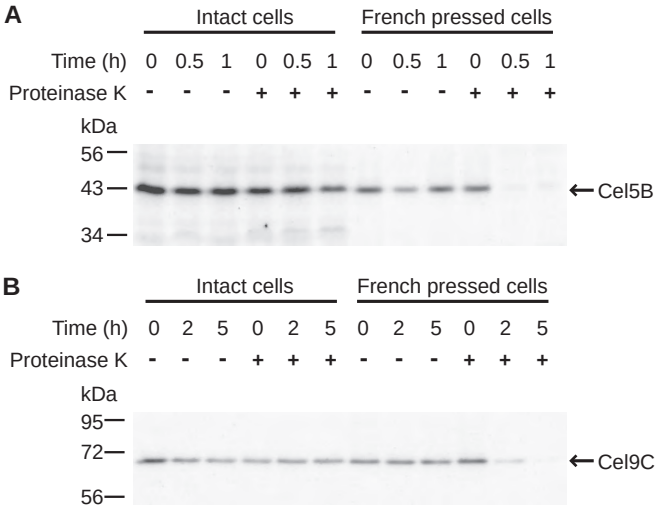

FIG 5 Effect of proteinase K treatment of wild-type cells on Cel5B and Cel9C. Proteinase K was added to the intact cells and French-pressed cells at a final concentration of 0.5 mg/ml (A) or 1.0 mg/ml (B), and samples were incubated at 25°C. Samples were removed at 0 h, 0.5 h, and 1 h (A) or 0 h, 2 h, and 5 h (B) for immunoblot analyses. The blot in panel A was probed with antibodies against Cel5B, and the blot in panel B was probed with antibodies against Cel9C. Samples that were not exposed to proteinase K (−) were also included. A higher concentration of proteinase K and longer incubation times were used in panel B, because in preliminary experiments, Cel9C in cell extracts was more resistant than Cel5B to digestion.

energy, it retained the ability to digest cellulose when allowed to grow to sufficient density using glucose as the carbon and energy source. This suggests that the mutant retained cell surface endoglucanases and perhaps other yet-to-be-identified cellulose-solubilizing and cellulose utilization proteins. Even cells lacking Cel5A, Cel5B, Cel5C, Cel9A, Cel9B, Cel9C, Cel9E, and Cel9F retained some ability to digest cellulose when provided with glucose (Fig. 6). Cel9D, the only remaining predicted endoglucanase, may be responsible for this cell surface cellulolytic activity. Cells of the Δ*sprP* mutant also grew on media containing glucose and filter paper but failed to digest the filter paper (Fig. 6). It is likely that some proteins required for crystalline cellulose utilization fail to be secreted in the Δ*sprP* mutant. Cel9D might be one of these proteins. The results suggest that the utilization of cellulose as the sole source of carbon and energy may require both cell surface and periplasmic endoglucanases.

TABLE 2 Processive ratios of *T. fusca* and *C. hutchinsonii* cellulolytic enzymes[a]

Enzyme	Processive ratio (SD)
T. fusca Cel6B (processive)	20 (0.06)
T. fusca Cel9B (nonprocessive)	0.54 (0.06)
C. hutchinsonii Cel5B	0.2 (0.01)
C. hutchinsonii Cel9C	0.05 (0.004)

[a] Each enzyme at a concentration of 1 μM was incubated with 1 mg/ml crystalline bacterial cellulose for 18 h. *T. fusca* enzymes were incubated at 50°C, and *C. hutchinsonii* enzymes Cel5B and Cel9C were incubated at 30 and 40°C, respectively. After incubation, the concentration of soluble and insoluble reducing sugars was determined. The processive ratio is defined as (micromolar concentration) soluble reducing sugar/(micromolar concentration) insoluble reducing sugar. SD indicates the standard deviation from the results from three reactions.

Cel5B and Cel9C are nonprocessive endoglucanases. While most endoglucanases are nonprocessive, several processive endoglucanases belonging to the glycosyl hydrolase 5 (GH5) and GH9 families have been identified (10, 11). The few processive GH9 endoglucanases known have CBM3 domains that maintain their contact with cellulose during processive digestion (11, 39, 40). Cel9C, as well as the other four *C. hutchinsonii* GH9 endoglucanases, lack obvious CBMs (1), suggesting that they are unlikely to be processive. The three *C. hutchinsonii* GH5 endoglucanases also lack obvious CBMs (1). Recombinant versions of Cel5B and Cel9C were expressed in *E. coli*, purified (see Fig. S8 in the supplemental material), and examined for their modes of action on crystalline bacterial cellulose. The ratios of soluble to insoluble reducing sugars produced by Cel5B and Cel9C were very low (0.2 and 0.05, respectively), as expected of nonprocessive enzymes, whereas the ratio for the known processive enzyme *T. fusca* Cel6B was 100-fold higher (Table 2).

Structure of *C. hutchinsonii* Cel5B. The structure of *C. hutchinsonii* Cel5B (PDB ID 5IHS) was determined to 1.1-Å resolution (Fig. 7). Initial phases were obtained by molecular replacement using the model of *Bacillus subtilis* Cel5A (41), which exhibits 50% sequence identity with *C. hutchinsonii* Cel5B. Cel5B possesses the $(\beta/\alpha)_8$ TIM-barrel topology typical of family 5 glycosyl hydrolases (Pfam accession code PF00150; Fig. 7A) (42–44). The root mean square deviation value from least-squares fitting of the *C. hutchinsonii* Cel5B model to that of *B. subtilis* Cel5A is 0.62 Å for 260 of 280 aligned Cα atoms, indicating that the core folds are essentially identical, although there are differences in the routing of some surface loops. The active site of Cel5B is also nearly

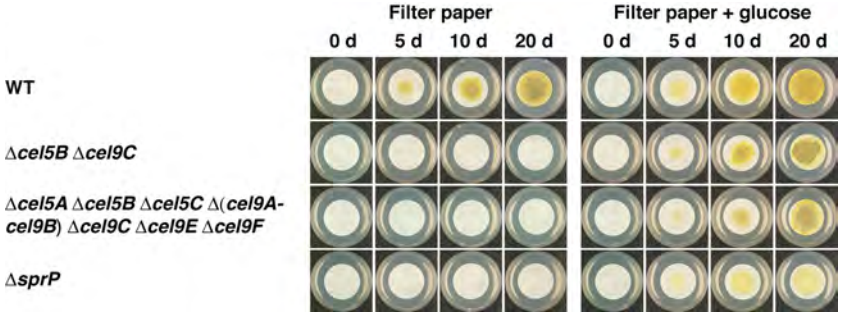

FIG 6 Cells lacking Cel5B and Cel9C digest cellulose when provided with glucose. Equal amounts of cells (3 μl; OD_{600}, 1.0) of wild-type and mutant strains were spotted on Whatman filter paper that was overlaid on Stanier agar or Stanier agar containing 0.1% glucose. Cells were incubated at 25°C. Clear zones indicate digestion of cellulose. The assay was performed in duplicate (with the same results), and one set of data is shown.

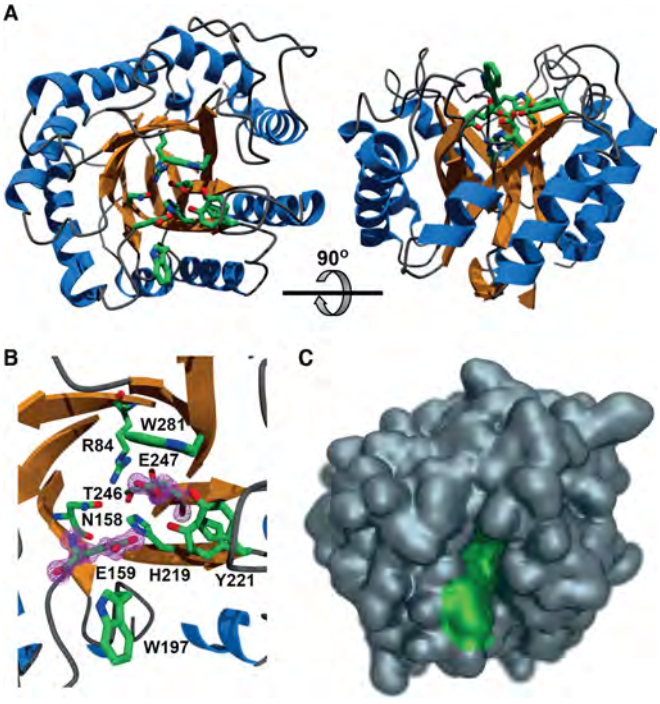

FIG 7 Crystal structure of *C. hutchinsonii* Cel5B. Orthogonal views of the Cel5B tertiary structure (A) show the $(\alpha/\beta)_8$- or TIM-barrel topology typical of GH5 family endoglucanases. Helices are shown in blue, β-strands in orange, random coil regions in gray, and active-site residues as sticks with carbon atoms colored green. (B) The closeup view shows the key active-site residues as sticks with green carbons. Representative $2|F_o| - |F_c|$ electron densities (magenta mesh) are shown for E159 and E247. (C) The surface of the enzyme is shown with the active site marked in green. The orientation is identical to the left half of panel A. This image was produced using the *POVScript+* (62) modification of MolScript (63) and rendered using POV-Ray.

identical to those of other family 5 glycosyl hydrolases, including *B. subtilis* Cel5A and *Hypocrea jecorina* Cel5A (PDB ID 3QR3) (45). Two amino acid residues of *C. hutchinsonii* Cel5B (E159 and E247) are predicted to be involved in the double-displacement mechanism characteristic of family 5 cellulases (Fig. 7B). In the *H. jecorina* Cel5A active site, a catalytic triad acts as a proton shuttle that allows a glutamate residue to protonate the glycosidic bond, promoting its cleavage (45). This catalytic triad is present in *C. hutchinsonii* Cel5B and consists of E159, the general acid/base catalyst, H219, and T246 (45, 46). E247 acts as a nucleophile that forms a covalent complex with the substrate. An extensive hydrogen-bonding network, including residues R84, Y221, and N158, maintains the E247 carboxylate in the correct orientation for catalysis. The two aromatic residues thought to be important for substrate binding and orientation (45) are also present in Cel5B as W197 and W281.

Processive exocellulases generally have tunnel-like covered active sites allowing the enzymes to remain attached to their substrates and processively release cellobiose as they move along the strands (47–50). In contrast, all nonprocessive endoglucanases that have been examined have open active sites (48, 51). These enzymes dissociate from their substrates after digestion and do not act processively. The surface of the *C. hutchinsonii* Cel5B in the vicinity of the active site is broad and relatively flat; the active site itself is located in a narrow channel in this surface (Fig. 7C). There are no structures that could be envisioned to clamp around a cellulose strand. The relatively featureless active surface of the enzyme, together with its monomeric quaternary structure, suggests that Cel5B has an open active site typical of nonprocessive endoglucanases.

DISCUSSION

Most well-studied cellulolytic bacteria use either cell-associated cellulosomes or soluble extracellular cellulases to utilize cellulose. Cellulosomes are composed of endoglucanases, cellobiohydrolases, CBMs, and other proteins (7). Endoglucanases and cellobiohydrolases function synergistically. Endoglucanases digest amorphous regions of cellulose generating free ends that are attacked by cellobiohydrolases to processively digest the adjacent crystalline regions, releasing cellobiose. β-Glucosidases complete the process by hydrolyzing cellobiose to glucose. Bacteria that use soluble extracellular enzymes instead of cellulosomes also typically rely on the synergistic action of endoglucanases and cellobiohydrolases to digest cellulose. A few of these bacteria use processive endoglucanases in addition to cellobiohydrolases (10, 11). The processivity of cellobiohydrolases is thought to depend on the structure near the active site of the enzyme. Cellobiohydrolases have tunnel-like covered active sites that allow them to remain attached to the cellulose strands as they processively digest them. In contrast, endoglucanases have open active sites and readily lose contact with their substrate after digestion (48, 51). The few known processive endoglucanases belonging to family GH9 (52, 53) have CBM3 cellulose-binding domains that are thought to tether the enzyme to a cellulose strand and allow movement along its length. Many aerobic cellulolytic bacteria also produce LPMOs that are thought to oxidatively cut cellulose strands in both crystalline and amorphous regions, generating additional sites for cellobiohydrolases to attack (12, 13).

C. hutchinsonii digests cellulose without releasing large amounts of extracellular soluble products (1, 24). Analysis of the *C. hutchinsonii* genome revealed the absence of genes encoding obvious cellobiohydrolases (1). Inspection of the CAZy database of carbohydrate-active enzymes indicates that *C. hutchinsonii* also lacks obvious LPMOs belonging to auxiliary activity (AA) families AA10, AA11, and AA13 (54). Since *C. hutchinsonii* actively digests crystalline cellulose without these enzymes, other novel proteins may be involved.

C. hutchinsonii has nine predicted endoglucanases. The construction of multiple-gene deletions allowed the roles of these proteins in cellulose utilization to be tested. A mutant lacking six of the nine predicted endoglucanases (Cel5A, Cel5C, Cel9A, Cel9B, Cel9E, and Cel9F) utilized filter paper, Avicel, and RAC as sole sources of carbon as well as did wild-type cells. Deletion of either *cel5B* or *cel9C* in this background, resulting in strains lacking seven of the nine predicted endoglucanases, had only a modest detrimental effect on cellulose utilization. However, the deletion of *cel5B* and *cel9C* from wild-type cells eliminated the ability to utilize cellulose as the sole source of carbon and energy. Cel5B and Cel9C are cell associated and appear to localize to the periplasm. The presence of at least one of these enzymes is required for utilization of cellulose as the sole source of carbon and energy.

It is unlikely that periplasmic endoglucanases can access crystalline cellulose. Instead, we suggest that cell surface endoglucanases digest amorphous regions of cellulose, and that yet-to-be identified proteins solubilize the cellodextrins and transport

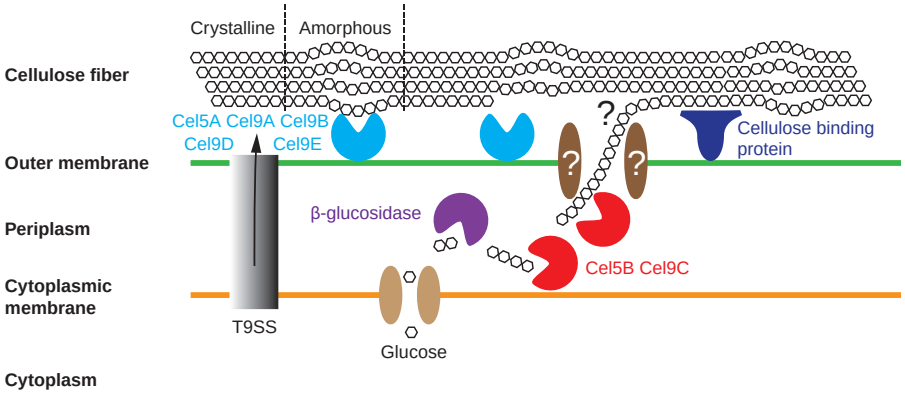

FIG 8 Model of *C. hutchinsonii* cellulose utilization. Cells attach to cellulose fibers using cellulose-binding proteins. Endoglucanases (Cel5A, Cel9A, Cel9B, Cel9D, and Cel9E, in light blue) predicted to be delivered to the cell surface by the T9SS cut cellulose within amorphous regions. Cellodextrins are transported across the outer membrane via a hypothetical cellodextrin transporter (brown). Further digestion occurs in the periplasm by Cel5B and Cel9C (red), releasing short oligomers, which are converted to glucose by β-glucosidases (purple). Glucose is transported across the cytoplasmic membrane.

them into the periplasm for further digestion. The requirement of periplasmic endoglucanases suggests that cellulose is not efficiently digested to cellobiose or glucose outside the cell, but rather that oligomers are transported into the periplasm. Cel9D is a likely candidate for an endoglucanase that could perform the initial extracellular digestion of cellulose. Cel9D is similar in sequence to periplasmic Cel9C (50% identical over 558 amino acids; see Fig. S7 in the supplemental material), but it has a CTD (Table 1) predicted to target the protein for secretion across the outer membrane by the T9SS, where it may be attached on the cell surface as a membrane-associated protein (33). Mutants that only retained two predicted endoglucanases, Cel9D and either of the periplasmic enzymes Cel5B or Cel9C, utilized cellulose as the sole source of carbon and energy, suggesting that extracellular or cell surface Cel9D may play an important role in cellulose utilization.

Recombinant Cel5B and Cel9C both exhibited properties of nonprocessive enzymes. The crystal structure of Cel5B supports this suggestion, since the predicted open active site is consistent with a nonprocessive mode of action. Our results indicating the lack of processivity of Cel5B and Cel9C contradict earlier reports (23, 38). The earlier studies lacked controls of known processive and nonprocessive enzymes and used amorphous cellulose (RAC) as the substrate in the processivity assays. Amorphous cellulose is not ideal for a determination of processivity. Nonprocessive endoglucanases often rapidly release large amounts of soluble cellodextrins and cellobiose from amorphous cellulose but fail to do so when crystalline cellulose is used as the substrate (28, 55). While Cel5B and Cel9C are not processive, additional studies are needed to determine if any of the other seven predicted endoglucanases exhibit processivity. Bioinformatic analyses, however, indicate that none of the predicted endoglucanases have family 3 CBMs, which are found on all known processive GH9 endoglucanases. Moreover, none of the predicted endoglucanases have any recognizable CBMs. This suggests that if *C. hutchinsonii* has processive endoglucanases, they must have a novel structure. In any event, extensive extracellular processive digestion of cellulose, which would produce large amounts of soluble sugars, such as cellobiose, is unlikely since it would be difficult to reconcile with the requirement of periplasmic endoglucanases for growth on cellulose.

In addition to lacking genes encoding predicted cellobiohydrolases, *C. hutchinsonii* also lacks genes related to those that encode LPMOs and swollenin (56), which also enhance the solubilization of crystalline cellulose. These observations suggest that novel *C. hutchinsonii* proteins may be involved in solubilizing crystalline cellulose. The proteins of unknown function encoded by genes near *cel5B*, *cel9C*, and *cel9D* are likely candidates, and the genetic tools available allow this to be tested. Several genes near *cel9C* have recently been demonstrated to be important for cellulose utilization, although their exact functions are not known (57, 58).

One property of *C. hutchinsonii* cells that may enhance their ability to digest cellulose is their active movement over surfaces by gliding motility (1, 21), a trait they share with many other members of the phylum *Bacteroidetes* (34). This movement may allow cells to reach sites on cellulose fibers with more amorphous regions and allow cell surface enzymes to sample many sites on a cellulose fiber. The gliding motility motor is powerful (59) and may also contribute more directly to the disruption and solubilization of crystalline cellulose.

In addition to proteins that solubilize crystalline cellulose, a mechanism to transport oligomers into the periplasm is needed if Cel5B and Cel9C are to gain access to their substrates. Many members of the phylum *Bacteroidetes* transport soluble oligosaccharides into the periplasm using proteins related to *Bacteroides thetaiotaomicron* starch utilization system proteins SusC and SusD (60). SusD is a cell surface starch binding protein, and SusC forms a channel through the outer membrane that is thought to allow energy-driven uptake of starch oligomers. Many *Bacteroidetes* have dozens of different SusC-like and SusD-like proteins that allow them to utilize diverse polysaccharides. *C. hutchinsonii* does not appear to use this system to utilize cellulose. It has two *susC*-like genes and two *susD*-like genes, but the deletion of all of these resulted in no defect in cellulose utilization (27). *C. hutchinsonii* may have a novel mechanism to transport cellodextrins into the periplasm.

The results reported here, in combination with those of others (22, 33), indicate that at least five of the nine predicted *C. hutchinsonii* endoglucanase-encoding genes (*cel5A*, *cel5B*, *cel9A*, *cel9B*, and *cel9C*) are expressed and produce functional endoglucanases. *cel9D* is also expressed (33) and likely produces a functional endoglucanase, since mutants that lack the other eight predicted endoglucanase-encoding genes maintained some ability to digest cellulose when they were provided with glucose.

A model for *C. hutchinsonii* cellulose utilization is shown in Fig. 8. Five endoglucanases (Cel5A, Cel9A, Cel9B, Cel9D, and Cel9E) are predicted to be delivered to the cell surface by the T9SS and perform the initial extracellular digestion of cellulose within amorphous regions. Experimental evidence supports the secretion and membrane localization of Cel5A, Cel9A, Cel9B, and Cel9D (22, 33). Cel9D may be essential for cellulose utilization, although further experiments are needed to determine this. The other cell surface endoglucanases shown are not essential but may perform semiredundant roles in this process. The model relies on yet-to-be-identified mechanisms to solubilize the cellodextrins and transport them across the outer membrane, where they are attacked by the periplasmic endoglucanases Cel5B and Cel9C. Four periplasmic β-glucosidases predicted by genome analyses (1) complete the digestion of cellobiose and short oligomers to glucose. The involvement of Cel5B and Cel9C in cellulose utilization is clear, but the proteins that are most critical for the other steps of the process remain uncertain. The genetic tools available for *C. hutchinsonii*, combined with biochemical analyses of proteins demonstrated to be important for cellulose utilization, provide a strategy to test this model. *C. hutchinsonii* uses an unusual approach to digest cellulose and is thus a potential source of novel proteins that may increase the efficiency of conversion of crystalline cellulose into soluble sugars and liquid biofuels.

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