APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1995, p. 4012–4015 Vol. 61, No. 11
0099-2240/95/$04.00+0
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Carbohydrate Transport by the Anaerobic Thermophile *Clostridium thermocellum* LQRI†

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Received 16 June 1995/Accepted 8 September 1995

Clostridium thermocellum **is an anaerobic thermophilic bacterium which degrades cellulose and ferments the resulting glucose, cellobiose, and cellodextrins predominantly to ethanol. However, relatively little information was available on carbohydrate uptake by this bacterium. Washed cells internalized intact oligomers as large as cellopentaose. Since cellobiose and cellodextrin phosphorylase activities were detected in the cytosol and were not associated with cell membranes, phosphorylation of carbohydrates occurred intracellularly. Kinetic studies indicated that cellobiose and larger cellodextrins were taken up by a common uptake system while glucose entered via a separate mechanism. When cells were treated with metabolic inhibitors including iodoacetate and arsenate, the uptake of radiolabeled glucose or cellobiose was reduced by as much as 90%, and this reduction was associated with a 95% decline in intracellular ATP content. A combination of the ionophores nigericin and valinomycin abolished the proton-motive force but only slightly decreased transport and ATP. These results suggested that the two modes of carbohydrate transport in *C. thermocellum* were ATP dependent. This work is the first demonstration of cellodextrin transport by a cellulolytic bacterium.**

Several anaerobic thermophiles depolymerize and ferment plant carbohydrates including cellulose and hemicellulose (10, 27, 28). Since some species metabolize the products to ethanol, there has been interest in the bioconversion of fibrous biomass to fuel alcohol by these anaerobes. *Clostridium thermocellum* is a cellulolytic thermophile which has been most often mentioned as a potential candidate for ethanol production (29). There has been a great deal of work describing the molecular and genetic details of the *C. thermocellum* cellulolytic system (5, 7), but other metabolic characteristics have not been adequately considered.

Although nutrient transport is an integral and necessary component of bacterial growth and metabolism, relatively few details regarding solute uptake by *C. thermocellum* and other anaerobic thermophilic bacteria are known. Cellulose hydrolysis by microorganisms yields water-soluble dextrins which are substrates for intra- and extracellular enzymes. The presence of internally located enzymes implies that oligomers are transported across the cell membrane. Several investigators have reported that glucose and cellobiose are taken up by active transport mechanisms (8, 9), but there is little information on the driving force for uptake. There is also some confusion as to whether phosphorylation participates in sugar uptake (19, 26). In addition, because of the lack of commercially available substrates, there has been almost no study of cellodextrin transport by bacteria. Since ethanol production from fibrous biomass can only proceed to the extent that the hydrolytic products of cellulose degradation are transported and metabolized, we investigated the specificity and energetics of carbohydrate uptake by *C. thermocellum*.

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† Published with the approval of the Director of the Kentucky Agricultural Experiment Station as journal article no. 95-07-144.

MATERIALS AND METHODS

Cell growth. *C. thermocellum* LQRI (ATCC 35609) was grown anaerobically in a medium containing (per liter) 3 g of NaCl, 2 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.02 g of $CaCl_2 \cdot 2H_2O$, 0.6 g of cysteine, 6 g of yeast extract, 1 mg of resazurin, and 4 g of Na_2CO_3. The pH of the medium was adjusted to 6.7 with NaOH and was maintained under a 100% carbon dioxide atmosphere. Carbohydrates were prepared as separate solutions and added after the medium was sterilized.

Radiolabelled cellodextrins. Mixed cellodextrins were prepared by acid hydrolysis of crystalline cellulose (15). Purified cellotriose, cellotetraose, and cellopentaose were then isolated by charcoal-celite chromatography (15). The purity of each cellodextrin preparation was confirmed by high-pressure liquid chromatography (HPLC) with a Dynamax 60Å NH_2 column (4.6 by 25 mm; Rainin, Woburn, Mass.). The isocratic mobile phase consisted of 65% acetonitrile and 35% H_2O at a flow rate of 0.75 ml/min and 370 lb/in^2, and the compounds were quantitatively measured with a refractive index detector. The purity of the oligosaccharides from the synthesis reactions was also monitored by thin-layer chromatography (TLC). Samples were spotted on TLC plates (Whatman 60Å silica gel, 0.25 μm thickness) and developed twice for 90 min in solvent (ethyl acetate-H_2O-methanol, 40:15:20). The plates were sprayed with 50% H_2SO_4 and oven dried (110°C for 20 min).

Crude cell extracts from *C. thermocellum* were prepared from a cell paste of cellulose-grown cultures. Cells were harvested, washed, and disrupted by sonication. Large cell debris was removed by centrifugation (15 min, 10,000 × *g*, 4°C), and the cell extract was concentrated sixfold to a concentration of 12 mg of protein per ml with a 10,000-molecular-weight-cutoff ultrafiltration membrane (PM-10; Amicon, Beverly, Mass.). The cellobiose and cellodextrin phosphorylase activities in the concentrated extract were 3.6 and 2.6 μmol/min/ml, respectively, on the basis of assays described by Alexander (3, 4).

Radiolabelled cellobiose and cellotriose were synthesized on the basis of a protocol developed by Ng and Zeikus (18). Concentrated crude extract (350 μl) was incubated in a reaction mixture (total volume, 1.35 ml) containing D-[U-^{14}C]glucose (0.10 mCi; 14.3 mCi/mmol), 82.1 mg of glucose-1-phosphate (disodium salt), 16.3 mg of Tris, and 2.7 mg of $MgCl_2 \cdot 6H_2O$. The mixture was incubated for 45 min at 60°C, and the reaction was terminated by boiling for 3 min. Precipitated protein was removed by centrifugation (13,000 × *g*, 10 min, 25°C), and the supernatant was applied to a charcoal-celite column as previously described (18). Radiolabelled cellobiose (7.2 mCi/mmol) and cellotriose (7.2 mCi/mmol) were eluted with 14 and 22% ethanol, respectively, and purity was confirmed by HPLC and TLC.

Radiolabelled cellotetraose and cellopentaose were synthesized by incubating 150 μl of concentrated crude extract in a reaction mixture (total volume, 500 μl) containing α-D-[U-^{14}C]glucose-1-phosphate (0.013 mCi; 2.87 mCi/mmol), 27.5 mg of cellotetraose, 30 mg of dithiothreitol, 0.27 mg of Tris, and 0.84 mg of EDTA (disodium salt). The mixture was incubated for 120 min at 60°C, and the reaction was terminated as described above. Labelled cellotetraose (0.06 mCi/mmol) and cellopentaose (1.65 mCi/mmol) were separated by HPLC.

Transport assays. Cells from exponentially growing cultures were harvested

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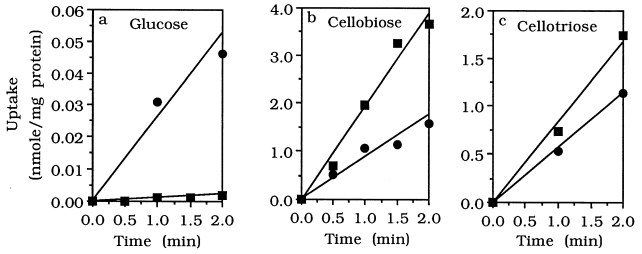

FIG. 1. Radiolabelled glucose (a), cellobiose (b), and cellotriose (c) uptake by *C. thermocellum* grown on glucose (●) or cellobiose (■). The assays were performed with 1.6 μM glucose, 103.2 μM cellobiose, and 59.6 μM cellotriose.

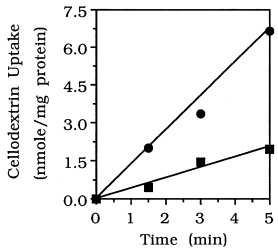

FIG. 2. Radiolabelled cellotetraose (●) and cellopentaose (■) uptake by *C. thermocellum* grown on cellobiose. The assays were performed with 1,720 μM cellotetraose and 150 μM cellopentaose.

under nitrogen (5,000 × *g*, 20°C, 10 min), washed twice with anaerobic buffer (50 mM potassium phosphate and 10 mM $MgCl_2$, pH 7.0), and resuspended to approximately 1 mg of protein per ml. Cell suspensions (1 ml) were dispensed into tubes which had been gassed with nitrogen, the cells were then warmed to 60°C, and uptake was initiated by the addition of radiolabelled carbohydrate. Stock concentrations of inhibitors were dissolved in either ethanol (tetrachlorosalicylanilide, nigericin, and valinomycin) or water (iodoacetate, sodium arsenate, and sodium fluoride) and were added to transport assays so that the addition volume was less than 5% of the total assay volume. Preliminary experiments demonstrated that ethanol or water additions had little effect (less than 5%) on transport. Samples (200 μl) were removed and transport was terminated by diluting the cells with 2 ml of ice-cold 100 mM LiCl and then immediately filtering them through a 0.45-μm-pore-size nitrocellulose membrane filter. The filters were dried (105°C, 20 min), and radioactivity was determined by liquid scintillation. Protein was measured by the method of Lowry et al. (11) after cell hydrolysis (0.2 N NaOH, 20 min, 100°C). Transport rates were based on initial rate determinations (<60 s) of duplicate incubations.

Bioenergetic measurements. Cells (approximately 1 mg of protein per ml) were harvested as outlined previously for transport studies. ATP content was determined by the luciferin-luciferase assay (12, 25). The proton-motive force (Δp) of cells grown on cellobiose was determined essentially as previously described (21), except that incubations were performed at 60°C. The intracellular pH was determined by accumulation in the cell interior of the weak acid [7-^{14}C]benzoate. Membrane potential was calculated from the uptake of [U-^{14}C]TPP, and the intracellular space was estimated by the differences in ^3H_2O (fully permeable probe) and [1,2-^{14}C]taurine (impermeable probe). The average cell volume was approximately 3 μl/mg of protein.

Internalization studies. The cells were prepared for transport studies as described previously. Resuspended cells (1 ml) were incubated with radiolabelled cellobiose (740 nCi; 7.2 mCi/mmol) or cellopentoase (250 nCi; 1.65 mCi/mmol). A portion of the assay mixture (900 μl) was layered onto 0.35 g of silicone oil (50:50 mixture of Dow Corning 550 and 556; Accumetric, Inc., Elizabethtown, Ky.) and centrifuged (13,000 × *g*, 3 min, 25°C). The aqueous phase and oil were carefully removed by aspiration, and the pellet was resuspended in 30 μl of 14% perchloric acid. The pellet was vigorously vortexed for 1 min and incubated at 4°C for 30 min. Acid-extracted pellets were then neutralized with 15 μl of KOH-$KHCO_3$ (2 M each) and centrifuged (13,000 × *g*, 30 min, 4°C) to remove salt and cellular debris. Preliminary experiments indicated that less than 5% aqueous extracellular contamination resulted from this procedure. Sugars in the neutralized extract were separated by TLC. Areas corresponding to bands of known sugars visualized in standard lanes were carefully scraped from lanes containing material from radioactive supernatants. The silica scrapings were mixed with scintillation fluid, and radioactivity was detected by liquid scintillation. All assays were typically done in duplicate.

Cell fractionation and phosphorylase assays. Cultures were grown on cellobiose (11 mM) and harvested during the logarithmic growth phase. Cells were washed and resuspended with 50 mM Tris-maleate-NaOH (pH 6.8) and disrupted by passage through a French pressure cell (1,200 kg/cm^2). Whole cells and large cell debris were removed by centrifugation (15,000 × *g*, 10 min, 4°C). The supernatant was then centrifuged at high speed (140,000 × *g*, 30 min, 4°C) to separate membrane fragments from soluble intracellular material. The membrane pellet was washed twice and resuspended in Tris-maleate-NaOH. Both fractions were assayed for cellobiose and cellodextrin phosphorylase activities (3, 4).

Data analysis. A Student's *t* test for paired values (22) was used to compare the effects of inhibitors on the rate of transport, ATP, and Δp.

RESULTS

Washed cells of *C. thermocellum* from growing cultures accumulated radiolabelled glucose and cellodextrins when assayed immediately after harvest (Fig. 1 and 2). Cells which were incubated in anaerobic buffer for 2 h at 60°C prior to the addition of radiolabelled sugars were unable to accumulate carbohydrates (data not shown). Glucose uptake was only observed in cells grown on the monomer, while cellobiose and cellotriose were taken up regardless of the growth substrate. In addition, cellotetraose and cellopentaose were transported by cellobiose- or glucose-grown cells (data not shown).

Carbohydrate uptake was generally reduced by metabolic inhibitors, and these compounds also had negative effects on Δp and ATP content (Table 1). A combination of nigericin and valinomycin reduced uptake and intracellular ATP only slightly but completely abolished Δp. Iodoacetate, an inhibitor of glycolysis, dramatically reduced the uptake of all sugars and decreased intracellular ATP levels by 90%; this large reduction in ATP formation was associated with a decrease in Δp. Other inhibitors of substrate-level phosphorylation, such as sodium fluoride and sodium arsenate, had similar effects on transport and the energy status of the cell (data not shown).

Since glucose was not transported by cellobiose-grown cells, it appeared that separate uptake mechanisms were used for these two sugars. This was confirmed by the observations that a 20-fold excess of unlabelled glucose did not inhibit the uptake of radiolabelled cellobiose and that unlabelled cellobiose did not affect glucose transport (data not shown). However, cellobiose uptake was virtually eliminated in the presence of a 20-fold excess of cellotriose, cellotetraose, or cellopentaose, and kinetic experiments indicated that these longer dextrins inhibited cellobiose uptake in a competitive fashion (Fig. 3;

TABLE 1. Effect of inhibitors on carbohydrate uptake and bioenergetic parameters of *C. thermocellum*[a]

Treatment[b]	Uptake[c] (%)					ATP (%)[d]	Δp (mV)
	G1	G2	G3	G4	G5		
Control	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]	61[a]
Tetrachlorosalicylanilide	83[b]	70[b]	59[b]	NT[e]	NT	80[b]	29[b]
Nigericin and valinomycin	64[b]	52[b]	59[b]	NT	NT	61[b]	0[c]
Iodoacetate	33[c]	10[c]	22[c]	36[b]	21[b]	10[c]	9[b,c]

[a] Values are means of two replicates. Values followed by the same letter(s) are not significantly different ($P < 0.13$) as determined by Student's *t* test.

[b] Cells were suspended in 50 mM potassium phosphate buffer (pH 7.0) supplemented with 10 mM $MgCl_2$. Tetrachlorosalicylanilide (10 μM), nigericin and valinomycin (10 μM each), and iodoacetate (400 μM) were added to cells at 60°C for 5 min prior to treatment.

[c] Uptake values are expressed as a percentage of the control. Uptake control values were 0.01, 1.24, 1.92, 1.30, and 0.41 nmol mg of protein^{-1} min^{-1} for radiolabelled glucose (G1; 1.6 μM), cellobiose (G2; 103.2 μM), cellotriose (G3; 59.6 μM), cellotetraose (G4; 1700 μM), and cellopentaose (G5; 153.0 μM), respectively.

[d] ATP levels are expressed as a percentage of the control (4.76 nmol of ATP per mg of protein).

[e] NT, not tested.

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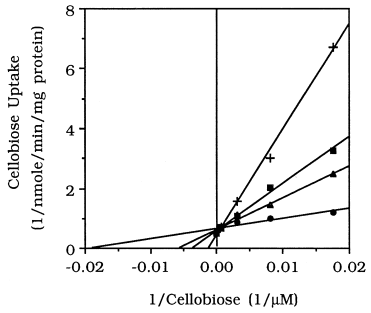

FIG. 3. Double-reciprocal plot for cellobiose uptake in the presence of cellopentaose at 0 (●), 53.5 (▲), 107 (■), and 214 (✚) μM. Cellobiose was varied from 52 to 621 μM.

similar plots for cellotriose and cellotetraose are not shown), with inhibition constants of 83, 21, and 21 μM, respectively. The transport of radiolabelled dextrins longer than cellobiose was inhibited by an excess of unlabelled cellobiose (data not shown), but kinetic experiments were not performed because of the limited availability of radiolabelled substrate.

Although cells accumulated radioactive sugars, it was not immediately apparent whether cellodextrins were taken up intact or were hydrolyzed extracellularly. Therefore, cells were incubated with radiolabelled sugars and were separated from the incubation buffer by centrifugation through silicone oil. Since preliminary experiments indicated that deenergized cells did not accumulate radiolabelled sugars in a time-dependent fashion (data not shown), the radioactivity associated with cells after passage through silicone oil did not appear to be due to nonspecific binding to the cell surface but rather to internal accumulation. Radiolabelled cellodextrins were detected in extracts of these cells (Fig. 4). Glucose was detected inside cells incubated with cellobiose, and this suggested that intracellular cleavage of the disaccharide was occurring as the incubation progressed. The smaller sugars detected after accumulation of cellopentaose may have resulted from reversal of cellobiose or cellodextrin phosphorylase activities and subsequent mixing of the radioactive label.

Fractionation of crude extracts revealed that most cellobiose and cellodextrin phosphorylase activity was associated with the cellular cytosol (Table 2). Small amounts of phosphorylase activities were found in the membrane fraction, but separate experiments indicated that this was apparently related to phosphate contamination in the assay mixture. High levels of phosphoglucomutase activity (57 nmol min^{-1} mg^{-1}) were observed in the cytosol fraction, while no activity was found in the membrane (data not shown); this result confirmed that the membrane fraction was essentially free of contamination by cytosolic enzymes.

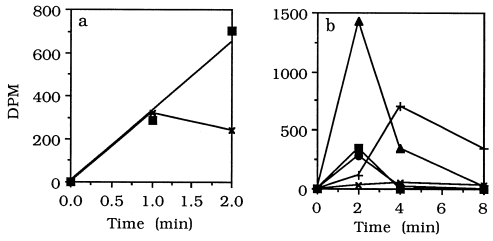

FIG. 4. Recovery of internal sugars in *C. thermocellum* incubated with radiolabelled cellobiose (a) and cellopentaose (b). Glucose (×), cellobiose (■), cellotriose (●), cellotetraose (✚), and cellopentaose (▲) were recovered after TLC separation of acid-extracted cells. DPM, disintegrations per minute.

TABLE 2. Cellodextrin phosphorylase activity of cell extract fractions from glucose- and cellobiose-grown cells

Growth substrate	Fraction	Sp act (nmol min^{-1} mg^{-1})[a]	
		Cellobiose phosphorylase	Cellodextrin phosphorylase
Glucose	Cytosol	150 ± 3	22 ± 2
	Membrane	23 ± 9	ND[b]
Cellobiose	Cytosol	151 ± 3	24 ± 1
	Membrane	15 ± 15	2 ± 1

[a] Activities were measured in duplicate, with the range indicated after the mean value.
[b] ND, not detected.

DISCUSSION

There are relatively few reports describing nutrient transport by anaerobic thermophilic bacteria. Sodium-dependent amino acid transport has been described for *Clostridium fervidus* (23), and glucose uptake appears to be mediated by facilitated diffusion in *Thermoanaerobacter thermosulfuricus* (6). There has been some confusion regarding the mechanism by which carbohydrates enter and are initially metabolized by *C. thermocellum*. It has been long known that the organism possesses cellobiose and cellodextrin phosphorylase (3, 4), and several investigators have stated that membrane-bound phosphorylase activities are involved in the translocation of these carbohydrates across the cell membrane (19, 20, 26). On the basis of recent work with nuclear magnetic resonance, it was suggested that cellobiose was phosphorylytically cleaved, presumably at the cell membrane, in a directional fashion so that glucose remained on the outside of the cell while glucose-1-phosphate was internalized (19); this type of transport by bacteria had not been previously described. Movements of protons across the membrane were also proposed to account for changes in internal pH supposedly associated with cellobiose versus glucose metabolism. However, no previous work had determined the location of phosphorylase activities. In addition, these models were based, in part, on the observation that extracellular glucose was sometimes detected when the organism was provided with cellobiose (19, 26). But this result may be accounted for by low levels of β-glucosidase activities which appear to be cell membrane associated (1, 2). In general, the evidence for membrane-based cellobiose cleavage and uptake appeared incomplete.

Our results clearly demonstrated that transport and cleavage of cellobiose and larger cellodextrins were not directly linked to each other. Cellobiose and cellodextrin phosphorylase activities were not associated with the cell membrane, and the presence of intact radiolabelled dextrins within *C. thermocellum* indicated that cellobiose and longer dextrins are internalized prior to phosphorylytic cleavage within the cell. There are several earlier reports of cellobiose transport in other bacteria (8, 13, 14), but this work is the first direct demonstration of cellobiose and cellooligomer (i.e., larger than cellobiose) internalization by a cellulolytic bacterium.

Earlier work with metabolic inhibitors provided presumptive evidence that ATP was required for glucose and cellobiose transport by *C. thermocellum* (9, 17), but there was no direct measure of energetic parameters associated with the inhibitory effects of such chemicals. Since substantial transport activities were observed in the absence of Δp (Table 1), it appeared that

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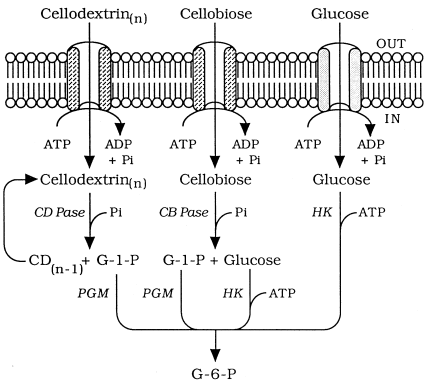

FIG. 5. Schematic model of carbohydrate uptake and phosphorylation by *C. thermocellum*. G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; Pi, inorganic phosphate; CB Pase, cellobiose phosphorylase; CD Pase, cellodextrin phosphorylase; HK, hexokinase; PGM, phosphoglucomutase.

transport was not dependent on electrochemical ion gradients. In contrast, transport inhibition was related to dramatic decreases in intracellular ATP levels. Although there were differences in the ability of tetrachlorosalicylanilide versus nigericin and valinomycin to decrease Δp, this may be due to a greater ability of the ionophore combination to enter and function in the cell membrane compared with that of the protonophore. Since intact cells were utilized in these experiments, internal metabolism may somewhat confound interpretation of the uptake studies. The use of membrane vesicles or metabolically defective mutants might provide a clearer answer. Nevertheless, the results generally support the hypothesis that ATP hydrolysis provides the driving force for glucose and cellobiose uptake. In addition, the presence of a common permease for cellobiose and larger cellodextrins demonstrates that longer oligomers also may depend on ATP for uptake.

The revised model for carbohydrate transport and utilization incorporates the elements of ATP-driven transport and internal phosphorylytic cleavage of intact β-linked carbohydrates (Fig. 5). If one assumes that an ATP molecule is required for uptake of either glucose or other larger carbohydrates, this model predicts higher yields on oligosaccharides compared with that on the monosaccharide. Past work with *C. thermocellum* did indeed show that higher yields (17) and lower maintenance energy coefficients were seen in cells grown on cellobiose versus glucose (24). Muir et al. (16) elegantly used maltodextrin metabolism by *Escherichia coli* to demonstrate that, from an energetic perspective, it was more efficient to take up an intact oligomer rather than cleave it extracellularly and transport the monomer sugar. Greater knowledge of transport mechanisms used by thermophilic organisms which degrade cellulose is required to better understand the overall process of organic fiber degradation and biomass conversion. Such information may be useful in developing rational schemes for manipulating the physiology of cellulolytic thermophiles and optimizing fermentation technology.

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