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Mode of Sugar Phosphorylation in *Clostridium thermocellum*

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ABSTRACT

Clostridium thermocellum uses cellobiose as a favored carbon source. After growth in cellobiose, cells cannot grow on glucose or fructose until they mutate to glucose or fructose utilization (*1*). This usually requires 180-200 h. One explanation for the initial lack of cell growth on monosaccharides is that cellobiose uptake and metabolism occur by a different phosphorylative mechanism than that for glucose or fructose, and the cells require a mutation to activate the new type of system. Our results show that there is no difference in the mode of phosphorylation of the three sugars. They all use an adenosine triphosphate-dependent mechanism rather than the phosphoenolpyruvate-phosphotransferase system.

Index Entries: *Clostridium thermocellum*; cellobiose; fructose; glucose; ATP; phosphoenolpyruvate-phosphotransferase system; sugar phosphorylation.

INTRODUCTION

Clostridium thermocellum is a thermophilic, anaerobic ethanol-producing organism with true cellulase activity and hence, a variety of biotechnological possibilities. This organism grows well on the disaccharide cellobiose,

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but shows a very long lag phase (180-200 h) when incubated with glucose or fructose (*1*). The cells that eventually grow represent mutants selected for their ability to metabolize the monosaccharides effectively. This long lag phase might possibly be the result of differences in sugar uptake.

Sugars can be transported into bacterial cells without undergoing any chemical change and can then be phosphorylated inside the cell, or they can be phosphorylated as part of the transport process. In most cases, the phosphoryl donor is either adenosine triphosphate (ATP) or phosphoenolpyruvate (PEP). ATP-dependent phosphorylation occurs after the sugar enters the cell, whereas the PEP-phosphotransferase system (PTS) is a process by which the sugar is phosphorylated as it is translocated. Clostridia are among the group of bacteria for which a PTS has been found. PEP-dependent conversion of fructose to fructose-1-phosphate has been reported in *Clostridium pasteurianum, Clostridium roseum, Clostridium rubrum,* and *Clostridium butyricum,* whereas it has not been detected in *Clostridium kluyveri, Clostridium tetanomorphum,* and *Clostridium formicoaceticum* (*2*). *Clostridium acetobutylicum* has a PTS for glucose (*3*). Patni and Alexander (*4*) reported a PTS in *C. thermocellum* for fructose and mannitol. There are two examples, in *Erwinia chrysanthemi* (*5*) and *Escherichia coli* (*6*), of cellobiose uptake occurring via a PTS.

The present work is directed at determining the modes of sugar phosphorylation in *C. thermocellum.* Since some literature suggests that a PTS is used for fructose in this organism, a working hypothesis is that the glucose and fructose are internalized by a PTS that must be induced or activated by mutation, whereas cellobiose is internalized and phosphorylated with ATP. This difference could then be responsible for the lack of growth of cellobiose-grown *C. thermocellum* when incubated with the monosaccharides until mutation occurs.

MATERIALS AND METHODS

Cell Growth and Extract Preparation

C. thermocellum ATCC 27405 (*7*) was grown in Fernbach flasks containing 2 L of chemically defined MJ medium as described previously (*1,8*). Cells in exponential phase were harvested by centrifugation in a Sorvall GS-2 rotor at 8500*g* for 15 min. The pellet was washed twice with 20 m*M* Tris-HCl buffer (pH 7.5) containing 1 m*M* dithiothreitol (DTT) and resuspended in 100 mL of the same buffer. All operations were carried out anaerobically at 4°C. *C. acetobutylicum* was grown as described by Gottwald and Gottschalk (*9*). The cells were broken by the method of Mimura et al. (*10*) with slight modifications. Cell suspensions in buffer were treated in a Bead-Beater homogenizer (Biospec Products, Bartlesville, OK) with 1:2 v/v cell suspension/glass beads (0.1 mm) in a 200-mL

metal chamber. Care was taken to remove as much air as possible from the chamber and the samples were homogenized for 3×2 min intervals, using the ice jacket provided with the instrument and allowing the suspension to cool for 5 min between each homogenization cycle. The cell extract was decanted from the glass beads and centrifuged at 5000*g* for 10 min to remove any unbroken cells and residual glass beads. The resulting cell-free extracts were kept on ice until used.

Assay for PEP-Dependent Phosphorylation

The assay for PTS was done using *C. thermocellum* cell-free extracts that were treated with toluene to render any vesicular structures permeable to charged substrates, as well as with untreated extracts. The assay was carried out with extracts from cellobiose-grown cells using ^{14}C-cellobiose, ^{14}C-glucose, and ^{14}C-fructose, with extracts from glucose-grown cells using ^{14}C-glucose, and extracts from fructose-grown cells using ^{14}C-fructose. As a positive control, glucose-grown *C. acetobutylicum* extracts, for which a glucose PTS is known to exist, were assayed using ^{14}C-glucose or ^{14}C-fructose (as a negative control). The assay procedure was as described by Mimura et al. (*10*). The assay mixture (0.1 mL) contained 24 m*M* Tris-HCl (pH 8.0), 1 m*M* DTT, 0.1 m*M* ^{14}C-sugar (~5 μCi/μmol), 5 m*M* $MgCl_2$, 10 m*M* KF, and the sample to be assayed, in the absence or presence of 10 m*M* ATP or 10 m*M* PEP. Mixtures were incubated at 60°C for 15 min (*C. acetobutylicum* was incubated at 37°C), the reaction stopped by immersion in ice-cold water, and the solutions filtered through DEAE filter disks (Whatman, Inc., Clifton, NJ; DE81) as described by Jacobson et al. (*11*). After 3×10 mL washes with water, the disks were air-dried and counted in standard toluene-based scintillation fluid. ATP- or PEP-dependent phosphorylation was calculated as the difference between mixtures containing and lacking the phosphoryl donor.

Preparation of ^{14}C-Cellobiose

^{14}C-Labeled cellulose (3.4 μCi/mg) was obtained from ICN Biomedicals, Costa Mesa, CA. The labeled cellulose (25 mg amounts) was treated with 10 mg crude *C. thermocellum* cellulase powder. The cellulase was allowed to bind to cellulose in a total volume of 4 mL containing 60 m*M* sodium succinate buffer, pH 5.8, and 10 m*M* $CaCl_2$ and DTT. After a period of 1 h, during which the tube was shaken intermittently, the unbound cellulase was washed off by centrifugation and the cellulose with the bound cellulase was resuspended in 4 mL of the same solution. The wash was repeated three times. With the removal of unbound cellulase from the cellulose, the probability of the formation of glucose from cellulose was drastically reduced owing to the removal of any β-glucosidase that might have been present in the crude enzyme (*12*). The cellulose-cellulase mixture was then sparged with N_2 and incubated at 60°C until

most of the cellulose had been broken down (5 d). The reaction mixture was centrifuged at 15,000*g* for 10 min and the supernatant collected.

Purification of ^{14}C-Cellobiose

The radiolabeled cellobiose was purified using the procedure of Ng and Zeikus (*13*) and Miller et al. (*14*). A Celite charcoal column was prepared by mixing 8 g each of Darco G-60 charcoal (MCB Manufacturing Chemists, EM Science, Gibbstown, NJ) and Celite 545 (Fisher Scientific Co., Pittsburgh, PA) in 40 mL of 2.5% stearic acid in absolute ethanol for 30 min, filtering, resuspending the adsorbent in 50% ethanol saturated with stearic acid, and finally resuspending in distilled water. Samples (1 mL) were applied to the column (2.6 cm × 4 cm) after it had been washed with several column volumes of distilled water to remove the ethanol. Glucose or other contaminating materials were removed by elution with 80 mL distilled water. Cellobiose was then eluted with 8% ethanol (v/v). Fractions with high radioactivity were pooled and concentrated by evaporation under vacuum at 40°C. The fraction was examined by high-performance liquid chromatography (HPLC) and found to contain only cellobiose. By this procedure, 1 mL of 1.2% cellobiose was obtained with a specific activity of 0.2 μCi/μmol.

PTS Enzyme I Activity

Enzyme I was assayed in cell extracts using the ^{14}C-PEP-pyruvate exchange assay (*15*). Assay mixtures (0.1 mL) contained 50 m*M* Tris-HCl (pH 7.5), 10 m*M* $MgCl_2$, 0.2 m*M* ^{14}C-PEP (12μCi/μmol), 2 m*M* pyruvate, 10 m*M* KF, 1 m*M* DTT, and the sample to be tested. Mixtures were incubated at 60°C for 30 min. The reaction was quenched by placing the samples on ice and adding 0.4 mL 0.02% 2,4-dinitrophenylhydrazine in 1*N* HCl. Tubes were incubated at 37°C for 10 min to allow reaction of pyruvate with dinitrophenylhydrazine. This solution was extracted by vortexing with 1 mL ethyl acetate; 0.6 mL of the upper organic phase was removed and counted in 5 mL Triton X-100/toluene scintillation fluid. Controls were prepared identically except pyruvate was omitted from the mixture.

PTS Enzyme II Activity

Isolated membranes of *C. thermocellum* grown on glucose and fructose were used and compared to membranes from *E. coli* grown in minimal medium (*16*) on glucose and fructose. Cell extracts prepared as described above were centrifuged at 4°C in an ultracentrifuge at 37,000 rpm for 90 min. The pellet was washed once in 20 m*M* Tris HCl (pH 7.5) containing 1 m*M* DTT and then resuspended in 0.5 mL of the same buffer. These membranes, stored at −80°C without loss of activity, were used in the transphosphorylation reaction. The assay mixture (0.1 mL) contained 0.1*M* potassium phosphate (pH 7.0), 1 m*M* DTT, 5 m*M* EDTA, 10 m*M* KF, 0.5 μ*M* ^{14}C-labeled sugar (300–400 μCi/μmol), and 1 m*M* sugar phosphate (fructose-1-phosphate when fructose was the labeled sugar and

Table 1
Mode of Sugar Phosphorylation in *C. thermocellum*

Carbon source for growth	Sugar added	Phosphoryl donor	Sugar-P formed (pmol/min mg protein)	Conclusion
Cellobiose	^{14}C-cellobiose	ATP	644 ± 50	ATP-dependent
		PEP	96 ± 20	
Cellobiose	^{14}C-glucose	ATP	14 ± 2	ATP-dependent
		PEP	0.7 ± 0.3	
Cellobiose	^{14}C-fructose	ATP	93 ± 4	ATP-dependent
		PEP	12 ± 2	
Glucose	^{14}C-glucose	ATP	12 ± 1.5	ATP dependent
		PEP	2 ± 1	
Fructose	^{14}C-fructose	ATP	404 ± 20	ambiguous
		PEP	232 ± 13	

glucose-6-phosphate when glucose was the labeled sugar). Mixtures were incubated for 1 h at 60°C, and the reaction stopped by placing the sample on ice. ^{14}C-Labeled sugar phosphate was determined by the method of Jacobson et al. (*17*). The reaction mixture was passed through a Dowex 1-X2 (Bio-Rad, Richmond, CA) ion exchange column. The negatively charged sugar phosphate binds to the column while the neutral sugar is eluted. The columns were washed with several volumes of distilled water. The sugar phosphates were eluted with 5 mL LiCl and counted in toluene-based scintillation fluid.

RESULTS AND DISCUSSION

The PTS was assayed based on the formation of sugar phosphate using ^{14}C-labeled sugar substrates in the presence of ATP vs PEP. Cellobiose-grown cells were separately tested for cellobiose, glucose, and fructose PTS's, whereas glucose-grown and fructose-grown cells were tested for glucose and fructose PTSs, respectively. The results are shown in Table 1. It is clear that the phosphoryl donor for cellobiose is ATP. This is in agreement with what is known about cellobiose internalization. It is believed that cellobiose is acted upon by a membrane-bound cellobiose phosphorylase that uses pyrophosphate to produce glucose, glucose-1-phosphate, and inorganic phosphate (*13,18,19*). In the presence of this enzyme, the initial uptake and phosphorylation do not require either ATP or PEP. The subsequent utilization of glucose and glucose-1-P, however, involves the expenditure of ATP (*20*). Although not much work has been done on cellobiose phosphorylation in other bacteria, there are reports of *E. coli* (*6*) and *E. chrysanthemi* (*5*) utilizing a cellobiose PTS. Both of these organisms are not known to have cellobiose phosphorylase activity.

Table 2
PTS Assay for *C. acetobutylicum* Grown on Glucose and Fructose and *C. thermocellum* Grown on Fructose

Organism	Carbon source for growth	Sugar added	Phosphoryl donor	Sugar-P formed (pmol/min mg DCW)
C. acetobutylicum	glucose	^{14}C-glucose	ATP	13 ± 2
			PEP	138 ± 10
	fructose	^{14}C-fructose	ATP	189 ± 4
			PEP	12 ± 1
C. thermocellum	fructose	^{14}C-fructose	ATP	365 ± 9
			PEP	127 ± 5

Glucose phosphorylation was also found to be ATP-dependent (Table 1). This confirms observations by Patni and Alexander (*21*) and Hernandez (*22*). The overall low values for sugar phosphate formed in this case are probably attributable to differences in the relative activities of the enzymes involved and in the K_m values for the three substrates. In the case of cellobiose-grown cells, ^{14}C-cellobiose (which had low specific activity) was used at a higher concentration to maximize the radioactivity in the assay. Therefore, it is possible that with glucose and fructose, the system is not saturated with the amount of sugar used.

The PTS is often induced by growth on the sugar, as observed for fructose in *C. pasteurianum* (*23*). When cellobiose-grown *C. thermocellum* cells were tested for fructose phosphorylation, the mode of phosphorylation was clearly ATP-dependent (Table 1). However, with fructose-grown cells, the mode of fructose phosphorylation was not clear. Sugar phosphorylation occurred with both ATP and PEP as phosphoryl donors, although the amount of sugar phosphate formed with ATP was twice that formed with PEP. To ensure that the assay conditions were allowing for proper determinations of PTS activity, another anaerobe, *C. acetobutylicum*, which has a documented glucose PTS, was examined. The conditions of the assay were identical except that the incubation temperature was 37°C, the optimum growth temperature for this organism. The results showed that in this organism, the PTS was used for glucose, whereas fructose phosphorylation was ATP-dependent (Table 2). Therefore, the assay conditions used were suitable for detection of PTS activity in *C. thermocellum*.

To determine without ambiguity whether fructose is transported by an inducible PTS in *C. thermocellum*, two additional assays were performed: examination of cell extracts for the presence of Enzyme I and Enzyme II of the PTS. Enzyme I is cytoplasmic and catalyzes a phosphoexchange reaction between PEP and pyruvate. Enzyme II is a sugar-specific membrane-bound enzyme that brings about the actual phosphorylation of the sugar, i.e., glucose to glucose-6-P and fructose to fructose-1-P (*24*). The results are shown in Tables 3 and 4. The level of Enzyme I activity as

Table 3
Assay for Enzyme I in *C. thermocellum* Extracts Grown on Different Sugars

Carbon source for growth	Carbon source added	^{14}C-pyruvate formed (nmol/min mg protein)
Cellobiose	Cellobiose	1.3 ± 0.15
Glucose	Glucose	1.4 ± 0.15
Fructose	Fructose	0.4 ± 0.07

Table 4
Assay for Enzyme II in Membrane Fractions of *C. thermocellum* Grown on Glucose and Fructose

Organism	Carbon source for growth	Sugar used	Sugar-P used	Sugar-P formed (pmol/h mg protein)
E. coli	Glucose	^{14}C-glucose	glucose-6-P	54 ± 5
	Fructose	^{14}C-fructose	fructose-1-P	13 ± 2
C. thermocellum	Glucose	^{14}C-glucose	glucose-6-P	0 ± 0.8
	Fructose	^{14}C-fructose	fructose-1-P	0 ± 0.6

reflected by the amount of labeled pyruvate formed from radioactive PEP was actually lower in cell extracts from fructose-adapted cells as compared to cells grown on cellobiose or glucose. All three values are similar and negligible compared to the Enzyme I activities of other bacteria with a PTS. *Streptococcus* sp. grown on glucose, for example, has a 10–15-fold higher Enzyme I activity (*10*) than reported here.

Enzyme II assays were performed with cell membranes from glucose- and fructose-grown cells. Positive cells of *E. coli* grown on glucose and fructose were used. From the data in Table 4, it is clear that fructose-grown cells of *C. thermocellum* do not possess any Enzyme II activity. The values observed for the *E. coli* control are comparable to those previously reported (*25*). Fructose phosphorylation is, therefore, ATP-dependent in *C. thermocellum*. This is contrary to the observations of Patni and Alexander (*4*) who reported a PTS for both fructose and mannitol. It should be noted that the strain used by them could readily utilize glucose, fructose, and mannitol and was probably a different strain (reported as strain 651) from the one used here (ATCC 27405). *C. thermocellum* ATCC 27405 is unable to use mannitol as a carbon source and can use glucose and fructose only after a long lag period. In addition, the assay of Patni and Alexander (*4*) detected the formation of fructose-1-phosphate in the presence of ATP and PEP.

All the data presented in this article indicate that there is no difference in the mode of phosphorylation of the three sugars and that it is ATP-dependent in all cases. Therefore, sugar phosphorylation cannot explain differences in growth of cellobiose-grown cells fed cellobiose vs glucose or fructose.

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