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Cellobiose Uptake and Metabolism by *Ruminococcus flavefaciens*

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The cellulolytic ruminal bacterium *Ruminococcus flavefaciens* FD-1 utilizes cellobiose but not glucose as a substrate for growth. Cellobiose uptake by *R. flavefaciens* FD-1 was measured under anaerobic conditions (N_2), using [G-^{3}H]cellobiose. The rate of cellobiose uptake for early- or late-log-phase cellobiose-grown cells was 9 nmol/min per mg of whole-cell protein. Cellobiose uptake was inhibited by electron transport inhibitors, iron-reactive compounds, proton ionophores, sulfhydryl inhibitors, *N,N*-dicyclohexylcarbodiimide, and NaF, as well as lasalocid and monensin. The results support the existence of an active transport system for cellobiose. Transport of [U-^{14}C]glucose was not detected with this system. Phosphorylation of cellobiose was not by a phosphoenolpyruvate-dependent system. Cellobiose phosphorylase activity was detected by both a coupled spectrophotometric assay and a discontinuous assay. The enzyme was produced constitutively in cellobiose-grown cells at a specific activity of 329 nmol/min per mg of cell-free extract protein.

Ruminococcus flavefaciens is among the most predominant cellulolytic bacteria isolated from the rumen of animals (5, 8). The physiology, enzymology, and genetics of cellulose degradation by this bacterium have been studied, but many questions remain unanswered. The cellulolytic enzyme system from *R. flavefaciens* has been examined in some depth and has been shown to be complex, involving numerous enzymes. The enzyme system exists in multiple forms that are distinguished by molecular mass (22) and are composed of exo-β-1,4-glucanase, endo-β-1,4-glucanase, and cellulodextrinase (10, 12, 22, 23). The main end products of cellulolysis by *R. flavefaciens* are cellotriose and cellobiose, with only small amounts of glucose being produced (23, 24). Although an aryl-β-glucosidase and cellobiose phosphorylase have been reported to be produced by this organism (1, 2), no extracellular β-glucosidase activity has been detected and the bacterium is unable to metabolize extracellular glucose (7, 23). *R. flavefaciens* FD-1 utilizes cellotriose and cellobiose but not glucose as substrates for growth (23) and initiates the Embden-Meyerhoff-Parnas pathway by cellobiose phosphorylase (1, 2). Therefore, *R. flavefaciens* is dependent on an uptake system for cellotriose and cellobiose when utilizing cellulose as a growth substrate.

However, although it is clear that intracellular metabolism of cellobiose is carried out by hydrolytic enzymes such as cellobiase or cellobiose phosphorylase, little is known of the actual mechanism for cellobiose transport. Cellobiose transport has been studied with *Clostridium thermocellum* (21), *Erwinia carotovora* (3), *Fibrobacter* (*Bacteroides*) *succinogenes* (20a), *Streptococcus bovis* (20), *Streptomyces granaticolor* (15), and *Thermomonospora curvata* (4). Evidence was found for both phosphoenolpyruvate (PEP):phosphotransferase system-dependent transport (21) and PEP:phosphotransferase-independent transport of cellobiose (3, 20). Kinetic measurements of cellobiose transport have only been done with *F. succinogenes* (20a) and *S. granaticolor* (15), and in these systems results indicated active transport systems.

The uptake of randomly tritiated cellobiose ([G-^{3}H]cellobiose) by the cellulolytic ruminal bacterium *R. flavefaciens* FD-1 was measured in this study. The use of [G-^{3}H]cellobiose is necessitated by lack of commercially available radiolabeled cellobiose. The characterization of cellobiose uptake and metabolism by an energy-dependent mechanism is reported herein.

(A preliminary account of these data has been presented at the Annual Meeting of the American Society for Microbiology 1989, New Orleans, La. [12a].)

MATERIALS AND METHODS

Organism and culture conditions. *R. flavefaciens* FD-1 was from the culture collection, Department of Animal Sciences, University of Illinois at Urbana-Champaign. The composition of the medium used was as described previously (9) except that cultures were grown with 0.1% (wt/vol) cellobiose; clarified ruminal fluid was eliminated, and the following additions were made: 0.00003% (wt/vol) 3-phenylpropionic acid, 0.000027% (wt/vol) phenylacetic acid, and 0.4% (vol/vol) B-vitamin mixture. The B-vitamin mixture used is a modification of previously described solutions (12, 18). The B vitamins were dissolved in double-distilled water and contained 0.02% (wt/vol) each calcium-D-pantothenate, lipoic acid, nicotinamide, pyridoxal-HCl, pyridoxamine, riboflavin, and thiamin-HCl and, at 0.001% each, p-aminobenzoic acid, biotin, folic acid, and vitamin B_{12}. The B-vitamin mixture was filter sterilized (0.22 μm), stored at 4°C in the dark, and added aseptically just prior to inoculation. Media were prepared by the method of Hungate (13) as modified by Bryant (6) under a 100% CO_2 atmosphere. Cultures were grown at 39°C, using a 2% inoculum from a culture in late log phase of growth. Growth was monitored by measuring the optical density at 600 nm (OD_{600}), using a Bausch and Lomb Spectronic 70 spectrophotometer.

High-performance liquid chromatographic purification of randomly tritiated [G-^{3}H]cellobiose. Randomly tritiated cellobiose ([G-^{3}H]cellobiose) was prepared by a catalytic exchange reaction (Amersham Corp., Arlington Heights, Ill.). The [G-^{3}H]cellobiose solution (25 ml; 1 mg/ml) was concentrated to dryness at 4°C, using a vacuum concentrator (Savant Instruments, Farmingdale, N.Y.). The residue was

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dissolved in 10 ml of 70:30 (vol/vol) acetonitrile-water. [G-^{3}H]cellobiose was purified by high-performance liquid chromatography, using a Bio-Sil Amino-5S column (250 by 4 mm; Bio-Rad, Richmond, Calif.) and a mobile phase of 70:30 (vol/vol) acetonitrile-water at ambient temperature. The injection volume was 0.25 ml and the flow rate was 1.0 ml/min, with fractions collected every 0.5 min. A single peak eluting at 4 to 7 min corresponded to the unlabeled cellobiose standard. Following each separation the column was washed with the mobile phase for 30 min. Radioactivity was measured with a liquid scintillation counter (Beckman LS TD5000; Beckman Instruments, Fullerton, Calif.), and disintegrations per minute were calculated by using the external standard channel ratio and a ^{3}H quench curve. Those fractions with the highest disintegrations per minute, typically those occurring at 7 min, were pooled, and acetonitrile was evaporated under a stream of N_2 and filtered through a 0.45-μm sterile filter. The level of radioactivity of the purified [G-^{3}H]cellobiose was 0.13 mCi/ml.

Transport assays. Cells were harvested anaerobically (96% CO_2, 4% H_2) by centrifugation (5,000 × *g*; 15 min; 22°C), washed once with 20 ml of anaerobic sodium phosphate buffer (50 mM $NaPO_4$ [pH 6.8], containing 5 mM $MgCl_2$, 50 mM KCl, and 1 mM dithiothreitol) (11), and then suspended in anaerobic phosphate buffer (95% N_2, 5% H_2) to an OD_{600} of 1.0. This corresponded to 0.11 to 0.18 mg of cell protein per ml of cell suspension. To test the effect of K^+ and Na^+ on cellobiose transport, anaerobic sodium phosphate buffer was modified to contain Na^+ or K^+ as the only monovalent cation. Whole-cell protein was determined by the procedure of Lowry et al. (19), with cell suspension samples diluted 1:1 with 2 N NaOH, boiled for 10 min, and left at room temperature overnight. Bovine serum albumin was used as the standard.

Sugar uptake reactions were carried out at 39°C with continuous flushing with 100% N_2. The standard uptake assay contained 0.75 ml of anaerobic sodium phosphate buffer and 1.0 ml of cell suspension. Carryover cellobiose (≤1 mM) was consumed during preincubation (39°C) under N_2 for 20 min. Uptake was initiated by adding a prewarmed anaerobic mixture (0.25 ml) of 1 mM [G-^{3}H]cellobiose (20 μCi/μmol; 0.1 mM final concentrations). Glucose uptake was measured under the same conditions except that 0.25 ml of 1 mM [U-^{14}C]glucose (20 μCi/μmol; 0.1 mM final concentration; ICN Radiochemicals, Irvine, Calif.) was substituted for cellobiose. After initiation, samples (0.1 ml) were withdrawn at specific time intervals and filtered through prewashed HAWP membrane filters (0.45-μm pore diameter; Millipore Corp., Bedford, Mass.) followed by 2.0 ml of 39°C anaerobic sodium phosphate buffer. Each filter was allowed to air dry and placed in 5 ml of Aquasol scintillation cocktail (DuPont Corp., Boston, Mass.), and radioactivity was measured. Disintegrations per minute were calculated by using external standard channel ratio and either a ^{3}H or a ^{14}C quench curve.

Incorporation of cellobiose or glucose radioactivity or both into ice-cold 10% trichloroacetic acid-precipitable cell fractions was determined by taking an additional 0.1-ml sample at the indicated times, extracting by the method of Hylemon et al. (14), followed by filtration and washing (2 ml of double-distilled water) of the samples. The filters were air dried and the radioactivity was determined as described above.

Metabolic inhibitors were dissolved in double-distilled water, ethanol, or dimethyl sulfoxide and were preincubated with cell suspension for 20 min at 39°C prior to initiation of uptake. Buffer additions were adjusted accordingly to accommodate the volume of the inhibitor added. Controls for metabolic inhibitors were assays containing only the solvent in which they were dissolved. Cellobiose uptake was expressed as a percentage of control activity measured after 9 min of uptake.

Cellobiose phosphorylase assays. Cell fractions for cellobiose phosphorylase assays were prepared as follows. Unless otherwise stated, cells were harvested aerobically by centrifugation (6,000 × *g* for 20 min at 4°C), as oxygen had little or no effect on cellobiose phosphorylase activity. Pellets were washed with 30 ml of 50 mM $NaPO_4$ buffer (pH 6.8, 4°C) and centrifuged (10,000 × *g* for 20 min at 4°C). The cell pellet was suspended in 3 ml of 50 mM $NaPO_4$ buffer (pH 6.8, 4°C) and then passed through a French pressure cell (1,054 to 1,266 kg/cm^2) twice. The cell preparation was then centrifuged (10,000 × *g* for 10 min at 4°C) to remove debris and unlysed cells. The supernatant fluid was centrifuged (288,489 × *g* for 30 min at 4°C) with a Beckman TL 100 table-top ultracentrifuge. The supernatant fluid from this procedure was termed the cell-extract fraction. The pellet from this centrifugation was suspended in 3 ml of 50 mM $NaPO_4$ buffer (pH 6.8, 4°C) containing 2 M KCl followed by centrifugation (288,489 × *g* for 30 min at 4°C). The supernatant fluid from this procedure was termed the cell membrane fraction. Protein concentrations were determined by using the 230- and 260-nm procedure described by Kalb and Bernlohr (16). Cell fractions were kept at 4°C on ice until required for cellobiose phosphorylase assays.

Cellobiose phosphorylase was assayed by both methods described by Schimz et al. (27). The standard enzyme-linked spectrophotometric assay mixture contained the following: 50 mM Tris-maleate–NaOH buffer (pH 6.8); 33 mM Na-K-PO_4 buffer (pH 6.8); 3 mM $MgCl_2$; 2.5 U of phosphoglucomutase, 1 mM ATP, 4 U of hexokinase, 1 mM NADP; 4 U of glucose-6-phosphate dehydrogenase and 20 μl of either cell extract or cell membrane fraction. The final volume for all assays was 0.5 ml and the change in A_{340} was read with a Gilford Response spectrophotometer. For direct assay of cellobiose phosphorylase, the reaction mixture contained, in a final volume of 5.0 ml: 50 mM Tris-maleate–NaOH, pH 6.8; 33.33 mM Na-K-PO_4, pH 6.8; 3.33 mM $MgCl_2$; 2.0 ml of double-distilled water; and 1 mM cellobiose. The reaction was started by adding 0.6 ml of either cell extract or cell membrane fraction. Samples (30 μl) were separated by two-dimensional thin-layer chromatography, using Kieselgel 60 F_{254} plates (20 by 20 cm) (EM Science, Gibbstown, N.J.) and the solvent system described by Schimz et al. (27). After thin-layer chromatographic separation in the two dimensions, plates were allowed to dry for 1 h before being sprayed with concentrated sulfuric acid (17). Standards, at 5 μg unless otherwise noted, were run and used as a visual comparison for concentrations of end products formed. The standards used were D-glucose, D-glucose-1-phosphate, D-cellobiose, D-glucose-6-phosphate, D-glucose-1,6-diphosphate, D-fructose-6-phosphate, and D-fructose-1,6-diphosphate.

PEP-dependent phosphorylation of cellobiose was measured by replacing hexokinase, ATP, and phosphoglucomutase with 10 mM PEP in the continuous assay described above. Glucose-6-phosphate formed by PEP-dependent phosphorylation and subsequent cleavage was measured by glucose-6-phosphate dehydrogenase-linked reduction of NADP.

Chemicals. [G-^{3}H]cellobiose was custom ordered from Amersham Corp. [U-^{14}C]glucose was purchased from ICN

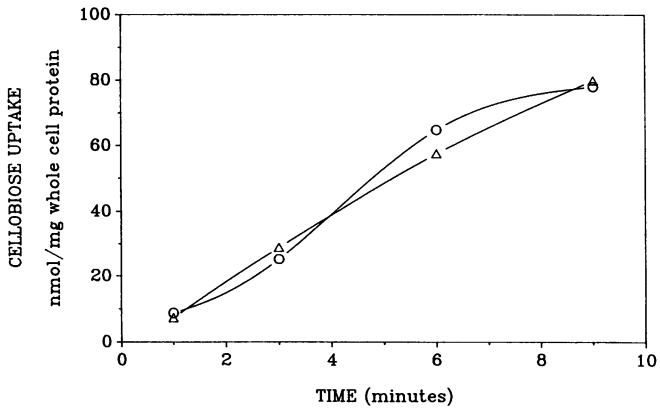

FIG. 1. Cellobiose uptake by early- or late-log-phase cells. Cells at either early (○) or late (△) log phase were suspended in buffer to an OD_{600} of 1.0. Cellobiose uptake was measured with 0.1 mM [G-^{3}H]cellobiose.

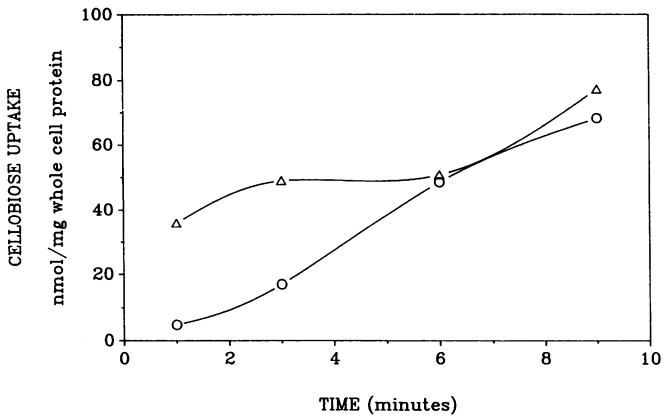

FIG. 2. Effect of cellobiose concentration on uptake. Late-log-phase cells were suspended in buffer to an OD_{600} of 1.0. Cellobiose uptake was measured with either 0.1 (○) or 1.0 (△) mM [G-^{3}H]cellobiose.

Radiochemicals. All metabolic inhibitors (except as noted), cofactors, hexokinase, phosphoglucomutase, glucose-6-phosphate dehydrogenase, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, Mo. The metabolic inhibitors potassium cyanide, sodium fluoride, menadione, dicoumerol, sodium azide, and mercuric chloride were reagent grade and obtained from commercial sources. All other reagents and chemicals used were of the highest grade commercially available.

RESULTS

Initially, cellobiose transport was measured with cells harvested from either the early (OD_{600} of 0.4) or the late (OD_{600} of 1.0) log phase of growth to determine whether culture (cell) age had an effect on the transport capabilities of *R. flavefaciens* FD-1. The pattern of cellobiose uptake by *R. flavefaciens* FD-1 cells harvested in early log phase of growth was similar to that by *R. flavefaciens* FD-1 cells harvested in late log phase of growth (Fig. 1). Cells were harvested anaerobically at 22°C as cells harvested either aerobically or at 4°C showed little or no ability to transport cellobiose. Furthermore, transport kinetics were identical regardless of the monovalent cation, either Na^+ or K^+, used in the transport and cell suspension buffers. Unless otherwise stated, all subsequent sugar uptake assays were measured with late-log-phase cells harvested anaerobically at 22°C in sodium phosphate buffer.

The effect of cellobiose concentration on cellobiose uptake was determined. Preincubated standard uptake assays were initiated with either 0.1 or 1 mM as the final cellobiose concentration (Fig. 2). Similar rates of uptake could be calculated from the endpoint of the 9-min assays for the two concentrations of added cellobiose, 8.25 and 7.20 nmol/min/mg of whole-cell protein, respectively. However, the kinetics of uptakes differed for the two concentrations of cellobiose tested. Biphasic kinetics were detected when the initial concentration of cellobiose was 1 mM. There was a greater rate of cellobiose transport in the first 1 to 3 min, followed by a period of reduced rate of transport for the remainder of the transport assay. That the kinetics of uptake were not linear over the entire time course of the assay when the initial cellobiose concentration was 1 mM suggests that transport may be limited by high concentrations of cellobiose-derived products which are feedback inhibitors of transport. Alternatively, the rate of cellobiose uptake could be masked by the eflux into the medium of radiolabeled fermentation end products. Therefore, unless otherwise stated, all uptake assays were measured with 0.1 mM as the cellobiose concentration, as the kinetics of transport were linear over the time course of the assay.

R. flavefaciens FD-1 cells were starved in anaerobic sodium phosphate buffer for an extended time period prior to cellobiose uptake experiments to determine the uptake capability of starved cells. Cells harvested at late log phase of growth were stable in anaerobic sodium phosphate buffer for as long as 3 h with little or no effect on uptake rates. With linear, reproducible conditions (variation of ±9.1%) being established using late-log-phase cells and a cellobiose concentration of 0.1 mM, all of the following experiments were compared with these conditions as the positive control. In addition, the first and last assay on each day was used as a positive control to check uptake capability.

It is known that *R. flavefaciens* cannot grow with exogenously supplied glucose, probably due to the inability to transport this sugar (7). As expected, *R. flavefaciens* FD-1 was not able to transport [U-^{14}C]glucose under conditions which permit reproducible cellobiose uptake. When equimolar amounts of [U-^{14}C]glucose and [G-^{3}H]cellobiose or unlabeled cellobiose were used in transport assays, [^{14}C]glucose transport was not detected. Indeed, it is interesting to note that cellobiose uptake decreased approximately 12% compared with controls with no glucose present. This level of inhibition is low and is near the experimental error for this system. However, the inhibition could be due to a low-affinity nonspecific binding of glucose by the cellobiose carrier protein, resulting in a low level of inhibition by glucose which would not be transported. Incorporation of [G-^{3}H]cellobiose into trichloroacetic acid-precipitable metabolites was not detected during the assay period of 9 min.

A variety of metabolic inhibitors were examined to determine the effect on cellobiose uptake, and the results are summarized in Table 1. Cellobiose uptake was inhibited by electron transport inhibitors, iron-reactive compounds, proton and metal ionophores, and sulfhydryl inhibitors. Furthermore, cellobiose uptake was abolished when cells were exposed to air for 20 min prior to use in transport assays.

Cell extracts and cell membrane fractions from *R. flave-*

TABLE 1. Effect of metabolic inhibitors on cellobiose uptake by *R. flavefaciens* FD-1

Class	Inhibitor (mM)[a]	Relative activity (% of control)[b]	% Inhibition
Control	None	100	0
	DMSO (5%, vol/vol)	70	30
	Ethanol (5%, vol/vol)	80	20
Electron transport inhibitors	Acriflavin* (1.0)	0	100
	Antimycin A† (0.05)	4	96
	Dicoumerol* (0.1)	72	28
	HOQNO (0.05)	51	49
	Menadione† (0.1)	17	83
Iron-reactive compounds	α,α-Dipyridyl† (1.0)	11	89
	o-Phenanthroline† (1.0)	0	100
	KCN (10)	34	66
Metal ionophores	Lasalocid† (0.01)	22	78
	Monensin† (0.01)	39	61
Proton ionophores	2,4-DNP* (0.4)	0	100
	CCCP† (0.05)	51	49
	FCCP† (0.05)	76	24
	Pentachlorophenol† (0.05)	64	36
Sulfhydryl inhibitors	*N*-Ethylmaleimide (0.5)	110	0
	$HgCl_2$ (0.5)	0	100
	pCMB* (0.5)	56	44
Miscellaneous	DCCD† (0.1)	48	52
	NaF (15)	2	98
	NaN_3 (10)	18	82
	O_2	4	96

[a] Inhibitors marked with an asterisk were dissolved in dimethylsulfoxide (DMSO); inhibitors marked with a dagger were dissolved in ethanol. All other inhibitors were dissolved in water. All inhibitors were compared against the appropriate solvent control. 2,4-DNP, 2,4-Dinitrophenol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; pCMB, *p*-chloromercuribenzoate; DCCD, *N,N'*-dicyclohexylcarbodiimide.

[b] Control value (no addition) was 9.0 nmol of cellobiose uptake/min/mg of protein.

faciens FD-1 were tested for the presence of cellobiose phosphorylase or the PEP-dependent phosphorylation of cellobiose or both. Cellobiose phosphorylase cleaves cellobiose with P_i as the phosphate donor, yielding equimolar amounts of glucose and glucose-1-phosphate, whereas PEP-dependent phosphorylation of cellobiose would yield glucose and glucose-6-phosphate after hydrolysis. Cellobiose phosphorylase was detected by using the enzyme-linked spectrophotometric assay in cell extracts (329 nmol/min/mg of protein) but not in cell membrane fractions. Direct assays of cellobiose phosphorylase as analyzed by thin-layer chromatography demonstrated the production of glucose and glucose-1-phosphate from cellobiose by both cell extract preparations and cell membrane fractions of *R. flavefaciens* FD-1. The amounts of glucose and glucose-1-phosphate produced by cell membrane fractions and detected by the direct assay were near the lower limit of detection after 6 h of incubation. Therefore, it is likely that in *R. flavefaciens* FD-1 cellobiose phosphorylase is a cytoplasmic enzyme that may be weakly membrane associated and may suggest a role for cellobiose phosphorylase as the terminal step in cellobiose transport. PEP-dependent phosphorylation of cellobiose by *R. flavefaciens* FD-1 was not detected under the conditions used.

DISCUSSION

The results reported herein support the existence in *R. flavefaciens* FD-1 of an active transport system for cellobiose which is not PEP dependent. In these experiments, cellobiose uptake by *R. flavefaciens* FD-1 was 180-fold higher than that reported for *S. granaticolor* (15), but were similar to those reported for *F. succinogenes* S85 (20a), 9 and 12 nmol/min/mg of whole-cell protein, respectively. Once transported, cellobiose is then cleaved by cellobiose phosphorylase. However, under the conditions used in these experiments, incorporation of [G-^{3}H]cellobiose into trichloroacetic acid-precipitable metabolites was not detected. This could reflect the nature of the radiolabeled substrate (^{3}H) rather than the actual fate of the transported cellobiose. During the time course of the assay, the ^{3}H label is probably removed by redox reactions and thus the majority is deposited on fermentation end products and not incorporated into cellular macromolecules, as would be seen for ^{14}C-labeled carbon skeletons.

The energy dependence of cellobiose uptake by *R. flavefaciens* is clearly evident by the effects of metabolic inhibitors. The inhibition of cellobiose uptake by electron transport inhibitors, iron-reactive compounds, proton and metal ionophores, and sulfhydryl inhibitors are consistent with sugar uptake by an energy-dependent system. However, these data cannot establish the specific energy source for uptake even though an electrochemical gradient appears to be involved.

In *R. flavefaciens*, carboxylation of pyruvate leads to succinate through the intermediates oxaloacetate and fumarate (7). Pyruvate also leads to acetylcoenzyme A, CO_2, and reduced ferredoxin, and acetate and ATP are produced by phosphotransacetylase and acetate kinase (7). Although *R. flavefaciens* does not contain cytochromes, many strains do contain ferredoxin (7). Thus, the formation of an energized transmembrane gradient is probably the result of ATP hydrolysis. The inhibition of cellobiose uptake by NaF and *N,N'*-dicyclohexylcarbodiimide is consistent with ATP involvement, even though at high *N,N'*-dicyclohexylcarbodiimide concentrations nonspecific inhibition of transport systems has been reported (28). Furthermore, the inhibition of cellobiose uptake by proton ionophores is also consistent with this hypothesis as these compounds are also known to dissipate ion gradients and thus the cellular ATP pool.

The effect of the metal ionophores monensin and lasalocid are also of interest as these compounds are known to dissipate proton gradients (25, 26). Although these data may suggest the involvement of Na^+ or K^+ in uptake, we could not detect any difference in cellobiose uptake when either Na^+ or K^+ was the sole monovalent cation added in the transport buffer. Additional work with membrane vesicles generated from *R. flavefaciens* is required to identify the exact mechanism of inhibition of cellobiose transport by lasalocid and monensin.

R. flavefaciens also grows rapidly with cellooligosaccharides as the carbon source (23), and as the final end products of extracellular cellulose hydrolysis are cellobiose and cellotriose (23, 24), it is reasonable to assume that a transport mechanism also exists for cellotriose. Whether this transport system is the same as that for cellobiose is unknown, but it is interesting to note that *R. flavefaciens* growth rates for cellotriose were threefold higher than growth rates for cel-

lobiose (23). This suggests that transport of cellotriose and phosphorolytic cleavage may be linked in a similar manner to that of cellobiose transport, thus representing a considerable increase in growth efficiency and ATP yield per hexose fermented. Further experiments are in progress to characterize cellotriose transport and metabolism and determine whether cellobiose and cellotriose are transported by the same or different systems.

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