Preferential Utilization of Cellobiose by *Thermomonospora curvata*

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***Thermomonospora curvata* was cultivated on mineral salts medium containing glucose and cellobiose under conditions that increasingly favored the uptake of glucose. In each case cellobiose was utilized in preference to glucose and induced β -glucosidase and endoglucanase activity. [^{14}C]glucose metabolism studies indicated that cellobiose was not cleaved by extracellular β -glucosidase and transported as glucose. No evidence of cellobiose phosphorylase or a cellobiose-specific phosphoenolpyruvate-phosphotransferase system was observed.**

In the enzymatic conversion of lignocellulosic biomass to fermentable sugars, the sugars repress production of cellulolytic enzymes and inhibit their activity (4, 20). Therefore, industrial strains that have a strong preference for uptake of cellulase inducer (cellobiose) over repressor (glucose) would be desirable. Here we demonstrate the marked preference of *Thermomonospora curvata* for uptake of cellobiose over glucose regardless of whether prior growth conditions favored glucose uptake.

MATERIALS AND METHODS

Organism and culture conditions. *T. curvata* (isolated from composting municipal waste at the Public Health Service-Tennessee Valley Authority Composting Project, Johnson City, Tenn.) (28) was grown at 53°C in minimal medium as described previously (29). Carbohydrates were autoclaved separately in distilled water and aseptically added after the salts had cooled. Cultures were grown in 1-liter Erlenmeyer flasks with 500 ml of medium shaken at 240 rpm at a 45° angle. One-hundred-milliliter portions were transferred to 250-ml flasks during early log phase prior to the addition of a second carbohydrate.

Enzyme assays. Samples were prepared for measurement of intracellular β -glucosidase (BG) activity by centrifuging 10 ml of the culture at $3,000 \times g$ for 10 min at 4°C. Five milliliters of the supernatant fluid was removed for assay of extracellular BG, endoglucanase (EG) activity, and residual carbohydrates. Cells were suspended in 0.1 M phosphate buffer (pH 7.0) with 10 mM dithiothreitol. The cells were lysed in a French pressure cell (3,500 lb/in²). Cell debris was removed by centrifugation ($20,000 \times g$, 25 min, 4°C).

In preliminary studies, we found that BG activity against *p*-nitrophenyl- β -D-glucopyranoside (PNPG) and cellobiose copurified through ammonium sulfate precipitation, size exclusion chromatography, and fractionation by high-pressure liquid chromatography (HPLC) on a DEAE anion-exchange column. This evidence agreed with previous reports (11, 12). We assumed that *T. curvata* extracts contained only one enzyme active against the two substrates. BG activity was routinely estimated in a reaction mixture consisting of 1 ml of 2 mM PNPG (Sigma Chemical Co.) in 0.1 M sodium acetate buffer (pH 5.6) and 20 μ l of cell extract. Incubation time was 10 min at 53°C. Two milliliters of 1 M sodium carbonate and 1 ml of distilled water were added to stop the reaction and enhance the color of the nitrophenyl (molar extinction coefficient of $2 \times 10^4 \text{ M}^{-1}$

cm^{-1} at 400 nm). Blanks were prepared in the same manner with 20 μ l of distilled water instead of cell extract. Enzyme units are expressed as micromoles of nitrophenyl released per minute.

The phosphorylated analog *p*-nitrophenyl- β -D-glucopyranoside-6-phosphate (PNPG-6-P) was synthesized by the procedure of Hengstenberg and Morse (13) and used to determine activity against phosphorylated substrates as indication of a cellobiose phosphoenolpyruvate-phosphotransferase system (PEP-PTS) or a cellobiose kinase system.

In an earlier study, extracellular cellulase activity against crystalline cellulose and carboxycellulose were proportional throughout growth (29). Therefore we used EG activity on carboxycellulose as a measure of cellulase in culture fluid as described earlier (30). Enzyme solution (0.1 ml of culture fluid) was combined with 1.9 ml of 4% carboxymethylcellulase (type 7L; Hercules Inc., Charlotte, N.C.) buffered with 0.1 M MES (morpholineethanesulfonic acid, pH 6.0). The reaction mixture was incubated for 10 min at 63°C. The concentration of reducing sugars was determined by the method of Bernfeld with glucose as the standard (2). EG units are reported as micromoles of glucose equivalents released per minute. Specific cellulase production (SCP) is reported as EG units per milligram (dry weight) of cells.

Carbohydrate concentrations were determined by HPLC. Solvent (HPLC-grade distilled water) was delivered by an Isco high-pressure syringe pump at 0.7 ml/min. Samples consisted of 6 μ l of clarified culture fluid. Separation occurred in a Bio-Rad Aminex HPX-87P column (300 by 7.9 mm) heated to 85°C. Glucose 1-phosphate (G-1-P) and glucose 6-phosphate were identified by the same system with a Bio-Rad Aminex HPX-87H column heated to 35°C with 0.008 N sulfuric acid at 0.7 ml/min. Sugars were detected with an Erma Optical Works ERC-7810 refractive index detector. Peak integration was performed on an LDC/Milton Roy CI-10 integrator. Peak heights were used to determine sugar concentrations by comparing samples with external standards. The average standard deviation, based on determinations from groups of three to eight replicates, was $\pm 7.6\%$.

Cellobiose phosphorylase (CBPase) was assayed by both the continuous and discontinuous methods of Schimz et al. (26). The continuous-reaction mixture contained phosphate buffer, 30 μ mol (pH 7.0); NADP, 0.5 μ mol; maleimide, 36 μ mol; ATP, 3.3 μ mol; MgCl_2 , 10 μ mol; glucose 6 phosphate dehydrogenase (Sigma Chemical Co.), 20 U; carbon source, 20 μ mol (glucose or cellobiose); and crude extract, 100 μ l in a total volume of 3.1 ml. Reaction mixtures were incubated at 37°C, and the OD_{340} was monitored to determine the rate

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FIG. 1. Glucose concentration (Δ) and cellobiose utilization ($\circ$) during exponential growth ($\square$) of *T. curvata* in cellobiose minimal medium.

of NADP reduction. A milliunit of activity was defined as 1 nmol of NADPH formed per min and compared with the activity observed with 15 U of phosphoglucumutase (Sigma Chemical Co.) present in the reaction mixture. In the discontinuous assay the reaction mixture contained 20 μ mol of phosphate buffer (pH 7.0), 20 μ mol of cellobiose, and 100 μ l of crude extract in a 1-ml volume. This mixture was incubated for up to 3 h at 53°C. Periodically, samples were removed and assayed by HPLC for the presence of phosphorylated sugars. Two-dimensional thin-layer chromatography of the reaction mixture was performed by the method of Schimz et al. (26). Silica gel 60 (Kieselguhr; F-254; 20 by 20 cm, aluminum) plates were spotted with 3 μ l of sample. The first dimension contained propanol-ethylacetate-water (7:1:4, vol/vol). The second dimension contained ethyl acetate-isopropanol-water (100:65:35). Runs were performed at room temperature, plates were dried overnight at 25°C, and sugars were detected with anisaldehyde reagent (18).

Protein determinations were performed by the Bradford method (3) according to Bio-Rad technical bulletin 8L-0275 with bovine serum albumin as the standard. Culture growth was estimated as increase in OD_{610} and converted to milligrams (dry weight) of cells per milliliter from a standard curve.

14 C utilization. Uptake and metabolism of [14 C]glucose (New England Nuclear Corp.) were measured by the method of Hobbie and Crawford (15) modified as follows. *T. curvata* cells were cultivated in mineral salts medium containing either 0.5% glucose or 0.5% cellobiose to the early exponential phase (before extracellular cellobiose could significantly hydrolyze cellobiose). Cells were aseptically filtered on Gelman GN-6 membranes, washed once with an equal volume of sterile mineral salts without sugar, and resuspended. In preliminary [14 C]glucose utilization experiments, the reaction mixture contained 50 mg of unlabeled glucose, 1.5 mg of cells, and 1 μ Ci of [14 C]glucose in 25 ml of mineral salts medium. In subsequent experiments the reaction mixture consisted of 3 mg of cells, 20 mg of unlabeled glucose, and 0.1 μ Ci of [14 C]glucose in 10 ml of mineral salts medium. The reaction mixtures were placed in 50-ml serum bottles, sealed with rubber septa from which plastic cups were suspended. Each cup contained an accordian-folded Whatman no. 1 chromatographic filter paper strip (2.5 by 5.0 cm). Cultures were incubated at 53°C on a rotary shaker, and growth was terminated by the addition of 0.3 ml of 5 N sulfuric acid through the septum by syringe. Phenethylamine (0.2 ml) was slowly added by syringe to the folded paper to adsorb CO_2 , and the vials were shaken for an additional 40

FIG. 2. (A) Time course of substrate utilization by *T. curvata* in mineral salts medium containing a mixture of 10 mM glucose and 10 mM cellobiose. Symbols: $\circ$, residual cellobiose concentration; Δ , residual glucose concentration; $\square$, extracellular BG activity (units per milliliter of culture fluid). (B) Growth and enzyme activity of *T. curvata* under the culture conditions described for panel A. Top graph: Intracellular BG specific activity (Δ), endocellulase activity (SCP; $\square$). Bottom graph: Dry cell mass ($\circ$), pH of the culture fluid ($\bullet$).

min at room temperature. The papers were removed and immediately placed in scintillation vials containing 10 ml of Omnifluor (New England Nuclear). Cells were filtered on Gelman GN-6 membranes, washed once with mineral medium, air dried, and placed in Omnifluor. The ^{14}C radioactivity associated with the cells and CO_2 was measured in Beckman LS-3150P liquid scintillation spectrometer.

RESULTS

Accumulation of glucose in the culture fluid was observed during growth of *T. curvata* in minimal medium with 10 mM cellobiose as the sole carbon source (Fig. 1). This accumulation peaked at about 5.7 mM, a concentration accounting for about one-fourth of the carbon in the initial cellobiose supply. Utilization of the accumulated glucose did not begin until the cellobiose supply became growth limiting. The presence of glucose during growth on cellobiose had no effect on BG activity. When a 10 mM pulse of glucose or 2-deoxyglucose was added to cells growing on 0.5% cellobiose, BG activity was unaffected (data not shown).

When *T. curvata* was grown on minimal medium with an initial 10 mM concentration of both cellobiose and glucose, only cellobiose was taken up (rate, 0.7 to 0.8 mM/h). Again, an increased glucose concentration in the medium was observed (Fig. 2A). This accumulation was apparently due to extracellular BG activity, as almost one-fifth of the total culture BG activity appeared in the extracellular fluid. When the cells were lysed by French press, 86% of the remaining BG was associated with the soluble fraction. The remainder was found in the debris.

Extracellular glucose did not repress the BG activity (Fig. 2). Ten to 12 mM glucose was present during the exponential phase of growth, yet BG activity increased during this time. The specific cellulase production (EG units per milligram of cells) increased during the early exponential phase and was not completely repressed until the end of the exponential phase. In a related experiment, cells were incubated in minimal medium containing 10 mM cellobiose or both 10 mM glucose and 10 mM cellobiose (Fig. 3). The amount of BG activity per milligram of cells was unaffected by the presence of glucose in the medium.

The response of cells to a given carbon and energy source would be greatly influenced by the conditions under which the cells were previously cultured (reviewed in reference 6). If cellobiose were the preferred substrate, its addition to a glucose-grown culture at relatively low concentrations would inhibit glucose uptake and induce catabolic enzymes. Therefore a 6.5 mM pulse of cellobiose was added to *T. curvata* cultivated on a minimal medium containing 28 mM glucose. After cellobiose addition, the following effects were

FIG. 3. Time course of SCP in cultures receiving 10 mM cellobiose (●) or 10 mM glucose and 10 mM cellobiose (○). Glucose-grown cells were starved for 75 min. Sugars were then added at time zero.

FIG. 4. Effect of a cellobiose pulse on enzyme production during growth of glucose-adapted cells. Arrows indicate when cellobiose was added. Top graph: Residual glucose concentration in medium (□, ●); residual cellobiose in medium (×). Bottom graph: Dry cell mass (○, ●); intracellular BG activity (△, ▲). □, ○, △, Pulsed culture; ●, ●, ▲, control.

noted: (i) cessation of glucose uptake after 1 h (1/3 generation time); (ii) establishment of a cellobiose uptake rate which reduced the extracellular concentration by about 0.7 to 0.8 mM/h; (iii) induction of BG biosynthesis, apparent within 1 h after cellobiose addition; and (iv) no detectable effect on growth rate (Fig. 4).

In subsequent experiments, we cultured *T. curvata* in medium containing progressively higher ratios of glucose to cellobiose (60 mM glucose and 6 mM cellobiose, 300 mM glucose and 6 mM cellobiose, and 600 mM glucose and 6 mM cellobiose). The uptake of cellobiose in the presence of glucose was found at the 300 mM glucose to 6 mM cellobiose ratio. The culture containing 60 mM glucose and 6 mM cellobiose had one-half the cellobiose utilization rate shown in Fig. 2A and 4, but no significant glucose uptake was observed. Only at 300 mM glucose and 6 mM cellobiose was any glucose uptake detected. Cellobiose uptake was 0.2 mM/h. At 600 mM glucose and 6 mM cellobiose, growth was one-fifth the normal rate, and the accumulation of extracellular BG made measurement of sugar uptake rates unreliable.

The evolution of $^{14}\text{CO}_2$ from ^{14}C -labeled glucose by *T. curvata* was used to determine the extent of glucose metabolism in the presence of cellobiose. Cells growing in 11 mM glucose were pulsed with an equimolar concentration of cellobiose (Fig. 5). The rate of $^{14}\text{CO}_2$ production was severely reduced 0.5 h after the addition of cellobiose to the medium. The rate of glucose metabolism was one-fourth of the rate observed without cellobiose. Growth was unaffected by the substrate change, as the initial and the final concentrations of cells were identical in both the pulsed and control cultures (0.3 mg/ml initial, 0.57 mg/ml final). In subsequent experiments cellobiose concentrations as low as 2 mM significantly slowed the rate of $^{14}\text{CO}_2$ production. The culture fluid from these washed-cell suspensions was assayed

FIG. 5. Evolution of $^{14}\text{CO}_2$ from $[^{14}\text{C}]$ glucose by washed-cell suspensions in glucose minimal medium (●) and glucose minimal medium pulsed with equimolar cellobiose (○) after 2.5 h (arrow).

for BG activity for 3 h after the addition of cellobiose. No extracellular BG could be detected; therefore, extracellular cleavage of cellobiose would be negligible.

We also tested the ability of cellobiose-grown *T. curvata* both to take up glucose (measured as ^{14}C radioactivity associated with the cells) and to use it as a carbon and energy source (measured as ^{14}C radioactivity of evolved CO_2) (Fig. 6). Both glucose-grown and cellobiose-grown cell suspensions accumulated the same amount of ^{14}C . The amount of ^{14}C present in evolved CO_2 was slightly less in cellobiose-grown cells than in glucose-grown cells during early incubation, but was nearly identical by 3 h. These results indicate that growth in cellobiose does not cause severe lasting repression of transport system constituents necessary for the uptake of glucose.

The preferential utilization of cellobiose might be explained if CBPase were present within the cell. The phosphorylytic cleavage of cellobiose yields equimolar amounts of glucose and G-1-P from cellobiose and P_i . In *T. curvata*, neither continuous nor discontinuous assays yielded detectable G-1-P, which would accumulate in the reaction mixture if CBPase were present. In the discontinuous method, HPLC analysis of the reaction mixture, after incubation times of up to 3 h, determined that the concentration of glucose had risen to 7 mM, with no G-1-P observed. The lack of G-1-P was qualitatively confirmed by thin-layer chromatography of the reaction mixture. The continuous assay determined that no additional NADP reduction was observed with phosphoglucomutase in the reaction mixture, again confirming that no G-1-P was present.

Another possible explanation for the preference of cellobiose over glucose is cellobiose transport by a PEP-PTS. Substrate transport in *Thermomonospora* species has not been studied. However, in other bacteria, the PEP-PTS yields disaccharides with a phosphorylated nonreducing glucose residue (14). This compound would also occur in the cell if cellobiose kinase activity was present (23). In the metabolism of cellobiose, the next reaction would be catalyzed by cellobiose-phosphate hydrolase (CBP-hydrolase) in either case. We synthesized the cellobiose analog PNPG-6-P. At 100 mM concentrations of PNPG-6-P in the standard BG assay mixture, the phosphorylated analog resulted in only 1/4 the activity of PNPG (0.06 versus 0.25 U/ml) assayed under identical conditions, with the same cellobiose-grown crude extract. If CBP-hydrolase was the major cellobiose-metabolizing mechanism, hydrolysis of the phosphorylated analog would have occurred more rapidly than with PNPG due to BG or low-level CBP-hydrolase. In either

case we have seen no indication that this system is the major source of cellobiose cleavage.

DISCUSSION

The addition of glucose to a microbial culture growing on other carbon sources usually inhibits utilization of those carbon sources; this effect is termed catabolite repression (19) and has been observed for a variety of sugars in various organisms. The addition of glucose represses EG production (1, 9, 21, 27). This effect has been shown in cellulose-grown *T. curvata*. Glucose and 2-deoxyglucose repressed EG production; production resumed only after the repressor was consumed (7, 31). In contrast, the generation of glucose during growth on cellobiose had no effect on the continuous uptake of cellobiose from the culture fluid. Glucose was not utilized until the cellobiose concentration dropped below 3 mM.

The lysis of cellobiose to glucose during fermentation was due to extracellular BG, which was released during the growth of *T. curvata* on cellobiose. This extracellular/cell-associated BG ratio is similar to that observed earlier for another thermophilic actinomycete (12). In that study, little of the cell-associated BG could be released by washing with a variety of agents, indicating that most of the enzyme was intracellular, not just cell bound. A qualitatively similar effect has been reported for the thermophilic cellulolytic anaerobe *Clostridium thermocellum* (22). In that study, glucose accumulation relative to cellobiose was much lower (1/14) and was only observed when cellobiose was in large excess (about 60 mM) of that needed for growth. When glucose-grown *C. thermocellum* was transferred to a medium containing equal concentrations of glucose and cellobiose, glucose was consumed at a faster rate than cellobiose, as opposed to *T. curvata*, which utilizes cellobiose regardless of its culture history or the phase of growth at which glucose was introduced.

Inhibition of glucose utilization has been reported for the cellulolytic anaerobe *Bacteroides succinogenes* (8); uptake of glucose was inhibited by 25 or 50% depending on whether the cellobiose was introduced after or 15 min before the glucose, respectively. The inhibition of glucose uptake was less than that observed here with *T. curvata*.

Cellulose degradation by *T. curvata* yields both cellulase inducer (cellobiose) and cellulase repressor (glucose). At growth-limiting concentrations for hydrolysis of cellobiose from cellulose, glucose represses EG biosynthesis (31), but in the presence of even low (3 mM) cellobiose concentrations, glucose is apparently not assimilated. In *T. curvata* BG might act as the metabolic "gate" through which

FIG. 6. Rates of $[^{14}\text{C}]$ glucose uptake and metabolism to $^{14}\text{CO}_2$ by *T. curvata* previously grown in either glucose (○) or cellobiose (△).

cellulase repressor passes. EG repression by glucose would occur only when glucose accumulates intracellularly through BG activity or through glucose uptake when the cellobiose becomes growth limiting. This may function as a control mechanism, as EG production and BG activity are inversely proportional within the cell.

Low extracellular BG activity was observed in our growth and substrate utilization studies. Therefore, the possibility existed that the cellobiose was hydrolyzed extracellularly while the glucose was metabolized at a parallel rate. The production of $^{14}\text{CO}_2$ from glucose in washed-cell suspensions was reduced to 1/4 of the initial rate by the addition of equimolar concentrations of cellobiose. The preference for cellobiose over glucose was apparently not due to inhibition of a common transport bonding site, since glucose depletion from the medium and $^{14}\text{CO}_2$ formation from [^{14}C]glucose continued for 30 to 90 min after cellobiose was added to the medium. If these two substrates were competing for the same binding sites, the inhibition of glucose utilization would be immediate.

In *Escherichia* and *Salmonella* species, the transport systems for glucose and phosphorylated glucose derivatives remain uninduced in the absence of exogenous glucose; glucose generated intracellularly was ineffective for the induction of the phosphotransferase proteins needed for uptake of it or its derivatives (5, 24). In *C. thermocellum*, glucose-adapted cultures utilized glucose in preference to cellobiose, but cellobiose-adapted cultures could not transport glucose; this inability resulted in a prolonged lag phase of 12 to 30 h after transfer to glucose-containing medium (10). In contrast, *T. curvata* was able to utilize the glucose with little lag phase observed in the uptake and metabolism of [^{14}C]glucose.

Organisms which contain a CBPase grow faster on cellobiose than glucose (16, 17), yet growth rates of *T. curvata* were identical on both sugars (culture generation times of 2.95 and 3.05 h, respectively). This is consistent with the absence of CBPase activity in *T. curvata*.

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