APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1987, p. 2505–2510
0099-2240/87/102505-06$02.00/0

Vol. 53, No. 10

Regulation of β-Glucosidase in *Bacteroides ruminicola* by a Different Mechanism: Growth Rate-Dependent Derepression

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Received 14 May 1987/Accepted 17 July 1987

***Bacteroides ruminicola* B_14, a predominant ruminal and cecal bacterium, was grown in batch and continuous cultures, and β-glucosidase activity was measured by following the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside. Specific activity was high when the bacterium was grown in batch cultures containing cellobiose, mannose, or lactose (greater than 286 U/g of protein). Activity was reduced approximately 90% when the organism was grown on glucose, sucrose, fructose, maltose, or arabinose. The specific activity of cells fermenting glucose was initially low but increased as glucose was depleted. When glucose was added to cultures growing on cellobiose, β-glucosidase synthesis ceased immediately. Catabolite repression by glucose was not accompanied by diauxic growth and was not relieved by cyclic AMP. Since glucose-grown cultures eventually exhibited high β-glucosidase activity, cellobiose was not needed as an inducer. Catabolite repression explained β-glucosidase activity of batch cultures and high-dilution-rate chemostats where glucose accumulated, but it could not account for activity at slow dilution rates. Maximal β-glucosidase activity was observed at a dilution rate of approximately 0.35 h^{-1}, and cellobiose-limited chemostats showed a 15-fold decrease in activity as the dilution rate declined. An eightfold decline was observed in glucose-limited chemostats. Since inducer availability was not a confounding factor in glucose-limited chemostats, the growth rate-dependent derepression could not be explained by other mechanisms.**

Cellulose is usually a major component of ruminant diets, but relatively few ruminal bacteria are actively cellulolytic. *Bacteroides ruminicola* is unable to grow on cellulose, but it does possess considerable carboxymethylcellulase activity and is able to utilize water-soluble cellodextrins (up to seven glucose units) (25). *B. ruminicola* is a common gut microorganism that can account for up to 19% of total counts in the rumen. Robinson et al. (24) also found that this species accounted for more than 35% of the isolates from the cecal contents of pigs.

Most cellulases generate cellobiose and cellodextrins rather than glucose (7), and β-glucosidase (EC 3.2.1.21) is the terminal enzyme yielding hexose. When *B. ruminicola* was incubated with a mixture of cellodextrins, cellobiose and cellotriose appeared but not glucose (25). These results suggested that transport and extracellular hydrolysis were rate limiting and that β-glucosidase was not extracellular. Other workers have implied that β-glucosidase activity can be the rate-limiting step in cellulose utilization by cellulolytic microorganisms (8).

Rumen cellulolytic bacteria are very sensitive to even modest declines in pH (27) and are inhibited when large amounts of starch are added to the ration (20). Because *B. ruminicola* is very resistant to low pH, it is currently being used as a recipient for acid-resistant cellulase genes (29). Success in creating a cellulolytic organism which can better resist low pH will hinge on cellulase gene expression as well as effective product (cellobiose) utilization. *B. ruminicola* is amylolytic, and it is well documented that lucrative energy sources can repress the synthesis of other degradative enzymes (22). The following series of experiments examined the regulation of β-glucosidase synthesis by *B. ruminicola* in batch and continuous culture. Results indicated that (i) cellobiose was not needed as an inducer, (ii) synthesis was catabolite repressed by energy sources yielding high growth rates, and (iii) at slow growth rates synthesis was controlled by a novel mechanism that we have termed growth rate-dependent derepression.

(This work was done by H. J. Strobel in partial satisfaction of the requirements for the M.S. degree from Cornell University, Ithaca, N.Y., 1987.)

MATERIALS AND METHODS

Cell growth. *B. ruminicola* B_14 (4) was obtained from M. P. Bryant, University of Illinois, Urbana. The basal medium contained (per liter): 240 mg of K_2HPO_4, 240 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4,000 mg of Na_2CO_3, 600 mg of cysteine hydrochloride, 22.1 mM acetate, 6.0 mM propionate, 2.4 mM butyrate, 0.81 mM isobutyrate, 0.68 mM each isovalerate, valerate, and 2-methyl butyrate, 0.5 g of yeast extract, and 1.0 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and was anaerobically prepared as described by Hungate (13). In certain cases, the basal medium was compared with a minimal medium that lacked yeast extract and Trypticase and contained vitamins (6) and microminerals (31). Carbohydrates were prepared as separate solutions under O_2-free N_2 and added to the basal medium after being autoclaved. Batch incubations were performed in either butyl rubber-stoppered serum bottles (125 ml) or gas-washing flasks (500 ml) which were continuously purged with O_2-free carbon dioxide. Continuous-culture experiments were conducted with a New Brunswick model F-1000 fermentor that had a 360-ml continuous-

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TABLE 1. β-Glucosidase activity of *B. ruminicola* B_14 grown on different carbohydrates[a]

Growth substrate	μ[b] (h^{-1})	Sp act (U/g of protein)	% of activity with cellobiose
Cellobiose	0.73 ± 0.05	520 ± 0.71	100
Mannose	0.76 ± 0.01	340 ± 0.71	65
Lactose	0.60 ± 0.01	286 ± 6.4	55
Sucrose	1.08 ± 0.02	63 ± 1.4	12
Fructose	0.96 ± 0.11	42 ± 6.4	8
L-Arabinose	0.89 ± 0.04	34 ± 4.2	6
Maltose	0.82 ± 0.06	29 ± 1.4	5
Glucose	1.18 ± 0.05	27 ± 3.5	5

[a] Determinations were based on duplicate incubations which were harvested at an optical density at 600 nm of 1.0 during exponential growth.
[b] μ, Maximum specific growth rate.

culture vessel. Dilution rates were changed in a random fashion to prevent the selection of slow- or fast-growing mutants. The culture vessel was purged with O_2-free carbon dioxide, and agitation was at 300 rpm. All incubations were done at 39°C and pH 6.7.

Cell harvest. In the batch experiments, 10-ml samples were withdrawn from incubation vessels with a hypodermic syringe, and optical density was measured at 600 nm (Gilford spectrophotometer model 260; cuvettes of 1-cm light path). Cells were removed from the medium by centrifugation (8,400 × *g*, 10 min, 4°C) and washed with 10 ml of 10 mM potassium phosphate buffer (pH 7.0). Washed cells were suspended in 1 ml of buffer and immediately assayed for β-glucosidase activity (see below).

Continuous-culture samples were not taken until more than 98% of the medium had turned over. Carbohydrate samples (4 ml) were removed from the culture vessel and immediately separated from cells in a microcentrifuge (13,000 × *g*, 5 min, 4°C). Cell samples (10 ml) were taken at the same time and centrifuged and washed as described above.

Osmotic shock procedure. Periplasmic proteins were removed by the method of Neu and Heppell (21) as modified by Hughes et al. (12). Alkaline phosphatase served as a periplasmic marker enzyme and was assayed as described by Garen and Levinthal (9). Osmotic shock fluid was washed and concentrated approximately 10-fold with phosphate buffer in an Amicon stirred cell (PM-10 membrane filter, 50-ml cell).

Enzyme assay. β-Glucosidase activity was measured continuously as the hydrolysis of *para*-nitrophenyl-β-D-glucopyranoside (PNPG) at 400 nm. The reaction mixture (3.05 ml) contained 3.33 mM PNPG and 25 μl of whole cells (approximately 4 to 80 μg of protein). Initial rates of PNPG hydrolysis (<10% of PNPG converted to product) were calculated by using the molar extinction coefficient of *para*-nitrophenol (10,718 M^{-1} cm^{-1}, pH 7.15) (16). Activities were expressed as micromoles of PNPG hydrolyzed per minute per gram of protein.

Analyses. Extracellular carbohydrate was measured with Anthrone reagent (1). Glucose was measured by hexokinase- (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase- (EC 1.1.1.49) linked assays (2). Cellobiose was estimated from the difference between total carbohydrate and glucose. Mannose was measured by a glucose assay which contained (per assay mixture) 2 IU of phosphomannose isomerase (EC 5.3.1.8) and 2 IU of phosphoglucoisomerase (EC 5.3.1.9). The protein content of whole cells was measured by the Bradford dye method (3). Cells (0.1 ml) were first hydrolyzed with 0.2 N NaOH (0.25 ml) at 100°C for 15 min.

RESULTS

Enzyme location and activity. When *B. ruminicola* B_14 was grown with cellobiose (6 g/liter), whole cells exhibited high β-glucosidase activity (>500 μmol of PNPG hydrolyzed per min per g of protein). However, activity was not detected in culture supernatants or osmotic shock fluid. Activity was reduced at least 88% when cells were grown on glucose, sucrose, arabinose, fructose, or maltose, but significant activity could be detected when cells were grown on mannose or lactose (Table 1). Maximum specific growth rates were considerably lower with cellobiose, mannose, and lactose than the other sugars, but enzyme activity was not always proportional to growth rate.

Repression and derepression. When cells were grown on cellobiose, the addition of glucose (6 g/liter) during logarithmic growth caused an immediate increase in growth rate from 0.67 to 0.97 h^{-1}; the increased growth rate was associated with repression of β-glucosidase synthesis (Fig. 1). Similar effects on growth rate and enzyme synthesis were seen when sucrose, fructose, maltose, arabinose, or mannose was added to cellobiose-grown cultures. Specific activity did not increase when cellobiose was added to cultures which were grown on any of the carbohydrates listed in Table 1, even though many were used simultaneously. Glucose-grown cultures had low β-glucosidase activity irrespective of the initial glucose concentration (Fig. 2). However, as glucose became limiting, activity increased even though cellobiose, a likely inducer, was not present. The specific activity increase did not occur until glucose was less than 0.20 g/liter. Similar results were obtained in minimal medium which did not contain yeast extract or Trypticase (data not shown).

The addition of 0.25 g of glucose per liter to cellobiose-grown cultures decreased the specific rate of cellobiose utilization (Fig. 3a), and this change was associated with an almost immediate decrease in β-glucosidase specific activity (Fig. 3b). When glucose was less than 0.15 g/liter, there was an increase in specific activity and resumption of rapid

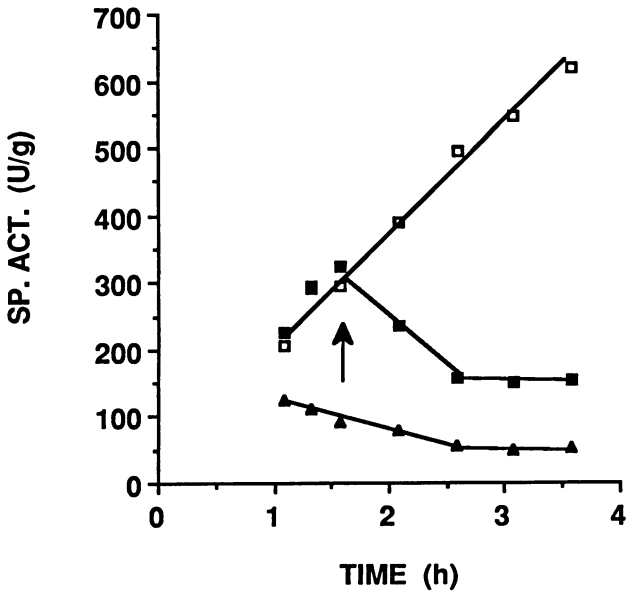

FIG. 1. Effect of glucose addition at 1.5 h on the specific activity of β-glucosidase in cellobiose-grown cultures (□), glucose-grown cultures, (▲), and cellobiose-grown cultures with glucose added (■).

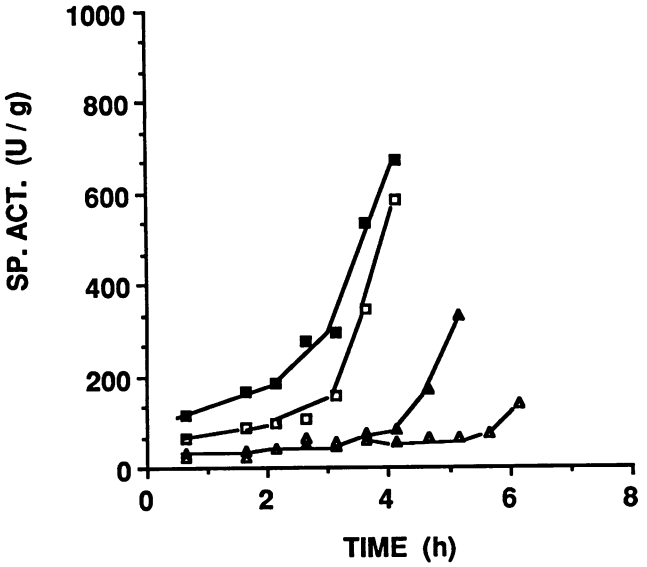

FIG. 2. Effect of initial glucose concentration on the specific activity of β-glucosidase. Glucose was present at 0.25 (■), 0.50 (□), 1.0 (▲), and 2.0 (△) g/liter.

cellobiose utilization. When cells were grown on mannose (2 g/liter), the growth rate was 0.67 h^{-1} and β-glucosidase activity was proportional to the increase in optical density (Fig. 4). However, addition of cellobiose (2 g/liter), a substrate also allowing high β-glucosidase activity (Table 1 and Fig. 3), increased the growth rate to 0.82 h^{-1}, and β-glucosidase synthesis was immediately repressed. When the specific growth rate eventually declined due to carbohydrate limitation, β-glucosidase synthesis was derepressed. Virtually identical results were obtained when the reciprocal experiment (2 g of mannose per liter in cellobiose-grown cultures) was performed. These results indicated that growth rate per se was more important than the specific carbohydrate supporting growth.

Continuous culture. When *B. ruminicola* was grown in glucose-limited chemostats (1 g/liter), β-glucosidase activity was repressed after glucose accumulated in the chemostat vessel at rapid dilution rates (Fig. 5). The effect of glucose accumulation was similar to that shown in Fig. 1 and 2. Maximal activity was obtained at a dilution rate of 0.38 h^{-1}, and at this dilution rate residual glucose was less than 0.01 g/liter. Glucose was as low or even lower at slower dilution rates, but β-glucosidase activity decreased at a nearly linear rate. As the dilution decreased from 0.38 to 0.10 h^{-1}, there was a nearly eightfold reduction in activity.

When *B. ruminicola* was grown in cellobiose-limited chemostats, cellobiose accumulated even at relatively slow dilution rates, but this accumulation had little effect on β-glucosidase activity (Fig. 5). Maximal activity was observed at dilution rates greater than 0.35 h^{-1}, and this activity was approximately twice that observed in the glucose-limited chemostats. Once again, β-glucosidase activity declined at slow dilution rates, and in this case there was a greater than 15-fold reduction in activity.

Effect of metabolic regulatory compounds. Cyclic AMP, dibutyryl cyclic AMP, imidizole acetic acid, or 3-indole acetic acid (each at 10 mM) was added to the basal medium to determine whether these low-molecular-weight compounds affected β-glucosidase synthesis. Glucose- or cellobiose-grown cultures (6 g/liter) were transferred at least three times in this medium to ensure that the compounds were present intracellularly. β-Glucosidase activities were not different from those observed in the absence of these compounds.

DISCUSSION

The genetic regulation of enzyme synthesis is extremely important to the survival of bacteria in natural environments, where energy sources are almost always limiting. Regulation not only prevents unnecessary protein synthesis; it also allows bacteria to select more lucrative substrates and maximize growth. Protein synthesis can be regulated at several levels, and lactose utilization by *Escherichia coli* is often used as a model for the coordination of regulatory mechanisms. However, surprisingly little is known about the regulation of enzyme synthesis in most other bacteria, especially ruminal species.

Lactose metabolism by *E. coli* is controlled by a variety of mechanisms, but enzyme induction (transcription in the presence of allolactose) is fundamental. Since the *lac* operon is only transcribed when inducer is present, wasteful protein synthesis is prevented when allolactose, and hence lactose, is absent. While induction allows enzyme synthesis in the

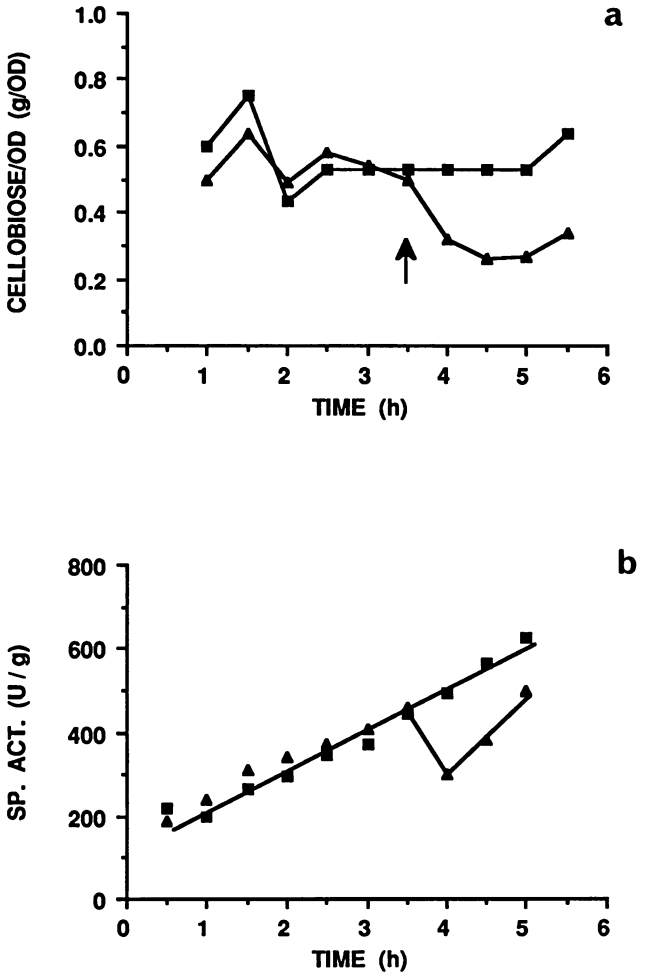

FIG. 3. Specific rate of cellobiose utilization (a) and β-glucosidase specific activity (b) by cellobiose-grown cultures (■) and cellobiose-grown cultures after glucose (250 mg/liter) addition (▲) at 3.5 h. OD, Optical density.

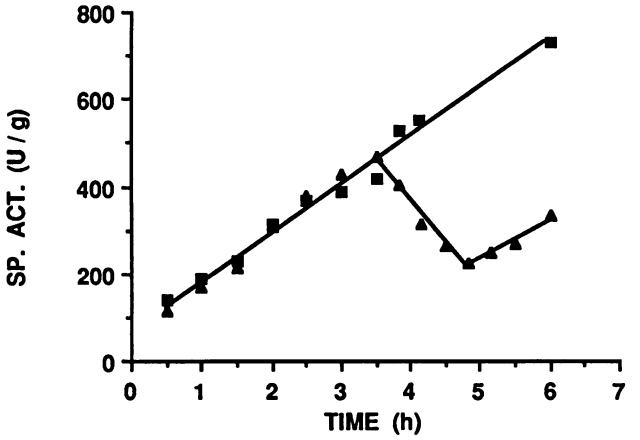

FIG. 4. Effect of cellobiose addition at 3.5 h on the specific activity of β-glucosidase in mannose-grown cultures (■) and mannose-grown cultures with cellobiose addition (▲).

presence of a specific substrate, many times an organism has the alternative of using one or more substrates. When glucose and lactose are provided, *E. coli* preferentially uses glucose before lactose. Glucose metabolism results in faster growth than lactose, and two regulatory systems allow the organism to use glucose but not lactose. One mechanism, catabolite repression, operates at the level of mRNA synthesis and represses transcription of the *lac* operon during glucose metabolism (22). This repression involves a drop in cyclic AMP concentrations and insufficient binding of cyclic AMP receptor protein at the site near the promotor. In general, substrates allowing fast growth are degraded by constitutive enzymes, while induced enzymes are catabolite repressed by these substrates (10).

Although catabolite repression results in preferential use of glucose, it alone cannot account for the immediate cessation of lactose uptake when glucose is added to lactose-utilizing cultures. Catabolite inhibition (also referred to as PTS repression) prevents the synthesis of induced enzymes by inhibiting the transport of inducer (or its precursor) into the cell (19). Since transport is inhibited, lactose uptake stops immediately. Thus, while catabolite repression directly represses mRNA synthesis, catabolite inhibition acts in a secondary fashion by preventing the uptake of inducer. This redundancy of control mechanisms (induction, catabolite repression, catabolite inhibition) ensures that *E. coli* only synthesizes additional protein if an appropriate substrate is present and only if a better substrate is unavailable.

Because β-glucosidase activity was sometimes high even when cellobiose was absent (Fig. 2), it appeared that cellobiose was not required as an inducer. The basal medium contained small amounts of Trypticase and yeast extract, and these complex ingredients could have contained an inducer. However, when these components were deleted, high activity was still observed in the absence of cellobiose. Since induction played a minor role, *B. ruminicola* regulated β-glucosidase synthesis by a less elaborate array of mechanisms than the *E. coli lac* operon. While the production of β-glucosidase in the absence of cellobiose might seem wasteful, this strategy is not necessarily inappropriate. Because cellulose is digested slowly by cellulolytic ruminal bacteria, small amounts of cellodextrins and cellobiose would almost always be available via cross-feeding. If β-glucosidase synthesis were dependent on induction, the initial rates of enzyme production might be insufficient for efficient cellodextrin or cellobiose utilization.

Since cellobiose utilization did not completely stop when glucose, the substrate allowing the fastest growth, was added to batch cultures (Fig. 3a), an inducer exclusion mechanism was probably not involved in regulation of β-glucosidase synthesis. Catabolite inhibition usually involves sugars which are transported by the PTS (23). *B. ruminicola* had negligible glucose PTS activity and nearly 40 times as much ATP-dependent phosphorylation of glucose (18).

Catabolite repression is, in its most basic sense, the inhibition of enzyme synthesis by better substrates, and it

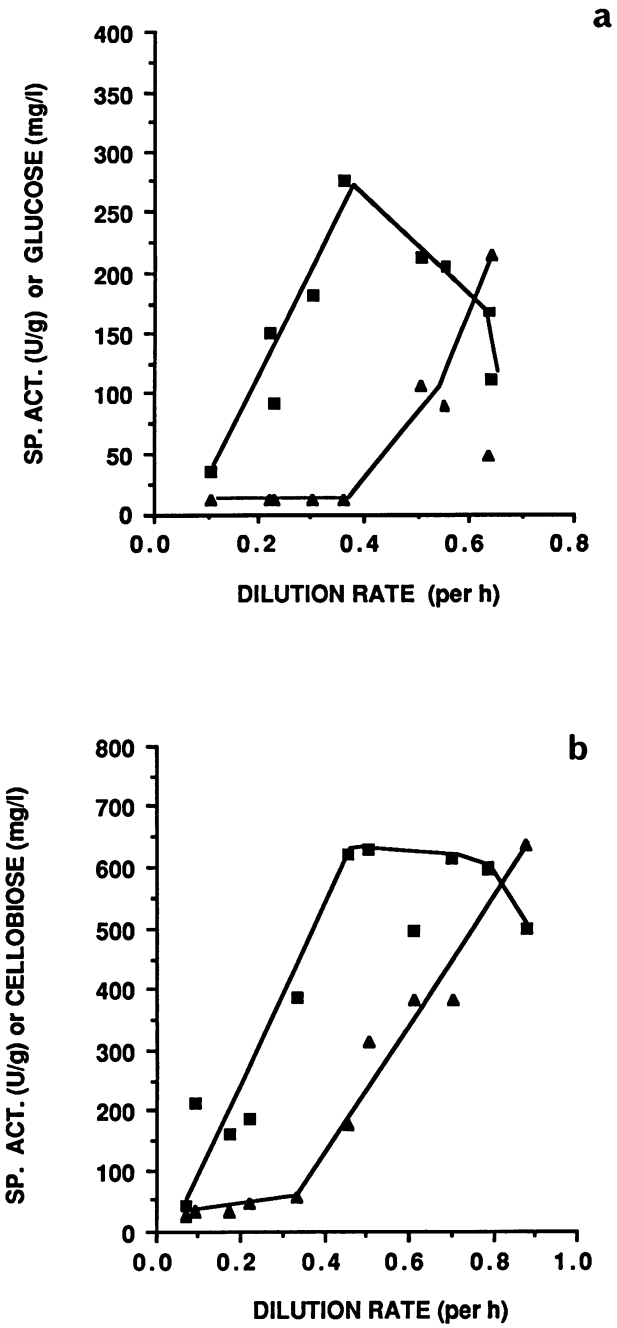

FIG. 5. Effect of dilution rate (growth rate) on the specific activity of β-glucosidase (■) and residual carbohydrate (▲) in (a) glucose- and (b) cellobiose-limited cultures.

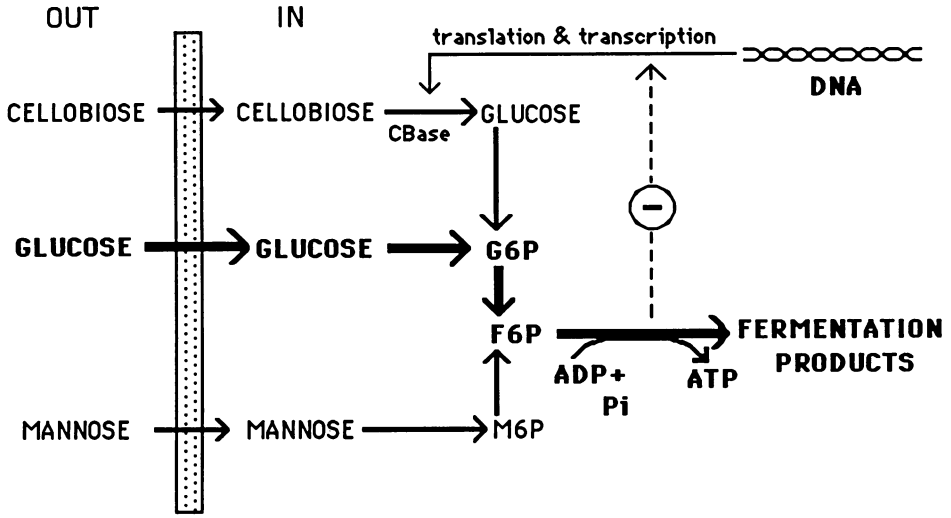

FIG. 6. Hypothetical scheme for catabolite repression of β-glucosidase synthesis by *B. ruminicola* B_14. The thickness of the arrows in the transport and glycolysis steps represents relative rates of flux. Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; M6P, mannose-6-phosphate; Pi, inorganic phosphate; CBase, cellobiase.

appeared to be an important factor controlling β-glucosidase synthesis. However, our observations showed that it differed in several ways from the classic catabolite repression of the *E. coli lac* operon. Although glucose was clearly a better substrate than cellobiose (Table 1), diauxic growth was not observed when glucose and cellobiose were both present (data not shown). Similar results were obtained when *B. ruminicola* was grown on a mixture of other carbohydrates (26). Also, cyclic AMP, a key component of catabolite repression in *E. coli*, and imidazole acetic acid, a possible substitute for cyclic AMP (17), had little effect on β-glucosidase synthesis. These results were consistent with the absence of cyclic AMP in other *Bacteroides* species (14).

The regulation of β-glucosidase synthesis in batch culture and at higher dilution rates in glucose-limited chemostats can be explained by the relationship of catabolic flux to catabolite repression. Substrates yielding the highest growth rates (e.g., glucose and sucrose) greatly repressed β-glucosidase synthesis. Growth on mannose, lactose, and cellobiose gave lower growth rates, and activity was much higher. However, a combination of mannose and cellobiose allowed a higher growth rate than either substrate alone, and the specific activity of β-glucosidase decreased. Since both of these sugars are metabolized by the Embden-Meyerhoff-Parnas pathway, it is likely that transport limited growth rate. Johnson et al. also noted an inverse relationship between enzyme production and growth rate in *Clostridium thermocellum* (15). Cellulase formation was repressed during rapid growth on cellobiose and derepressed during lag phases of batch culture growth. Similarly, amylase activity in the ruminal bacterium *Bacteroides amylophilus* was greatly reduced in nitrogen-limited chemostats when maltose was in excess (11).

Since cyclic AMP, the regulator of catabolite repression in *E. coli*, had little effect in *B. ruminicola*, other signals must be involved (Fig. 6). Because the concentrations of intracellular metabolites are sometimes related to the rate of sugar influx (28), an intermediary metabolite might serve as a signal controlling transcription. Influx of sugar, as indicated by growth rate, was greater for glucose than cellobiose or mannose, and the combination of mannose and cellobiose allowed greater growth rate than either one alone. Experiments are under way to test this hypothesis.

Although catabolite repression could account for the results in batch culture or rapid-dilution-rate chemostats in which glucose accumulated, it could not adequately describe continuous cultures that were severely energy limited. At low dilution rates, β-glucosidase activity was directly proportional to growth rate, and it appeared that protein synthesis was responding to a growth rate-dependent derepression mechanism. Small increases in specific activity were previously noted for amidase in *Pseudomonas aeruginosa* (5) and chondroitin lyase in *Bacteroides thetaiotaomicron* when dilution was increased (30). However, both of these enzymes were inducible, and an increased dilution rate could have increased the availability of inducer (34). Since β-glucosidase synthesis by *B. ruminicola* was not under inductive control, the derepression in glucose-limited chemostats was not confounded by inducer availability, and specific activity increased nearly eightfold. To our knowledge this latter mechanism for regulating protein synthesis has not been reported previously.

In wild-type strains of *E. coli*, β-galactosidase synthesis varies approximately 18-fold (33), but synthesis does not change significantly as a function of growth rate (32). When *B. ruminicola* was grown in cellobiose-limited chemostats, there was an approximately 15-fold decrease in β-glucosidase activity as the dilution rate decreased from 0.48 to 0.05 h^{-1}. This growth rate-dependent derepression allows the organism to match the rate of substrate availability with enzyme synthesis. Such a mechanism could be of great advantage to organisms growing in energy-limited habitats.

ACKNOWLEDGMENT

This research was supported by the U. S. Dairy Forage Research Center, Madison, Wis.

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