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Glycogen Biosynthesis via UDP-Glucose in the Ruminal Bacterium *Prevotella bryantii* B_14†

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***Prevotella bryantii* is an important amylolytic bacterium in the rumen that produces considerable amounts of glycogen when it is grown on maltose. Radiolabel studies indicated that glucose-1-phosphate was converted to UDP-glucose, and this latter intermediate served as the immediate precursor for glycogen synthesis. High levels of UDP-glucose pyrophosphorylase activities (>1,492 nmol/min/mg of protein) were detected in cells grown on maltose, cellobiose, glucose, or sucrose, and activity was greatly stimulated (by approximately 60-fold) by the addition of fructose-1,6-bis phosphate (half-maximal activation concentration was 240 μM). However, ADP-glucose pyrophosphorylase activity was not detected in any of the cultures. Glycogen synthase activity in maltose-grown cultures (48 nmol/min/mg of protein) was higher than that in cellobiose-, sucrose-, and glucose-grown cultures (<26 nmol/min/mg of protein). This is the first report of a bacterium that exclusively uses UDP-glucose to synthesize glycogen. The elucidation of this unique glycogen biosynthesis pathway provides information necessary to further investigate the role of bacterial glycogen accumulation in rumen metabolism.**

Glycogen, a branched glucose polymer, is a common storage energy compound in both prokaryotic and eukaryotic cells (2, 25, 26). Mechanisms of glycogen formation have been best studied for *Escherichia coli* (24–26). Glucose-1-phosphate is the early precursor for glycogen synthesis, and it is first converted to ADP-glucose in a reversible reaction catalyzed by ADP-glucose pyrophosphorylase (EC 2.7.7.27). The glucosyl unit of ADP-glucose is transferred to the nonreducing end of an α-1,4-glucan chain by glycogen synthase (EC 2.4.1.21), and α-1,6 linkages are generated by a branching enzyme (EC 2.4.1.18). UDP-glucose is considered to be the glucosyl donor for glycogen synthesis in mammalian cells (2, 19). Either ADP-glucose or UDP-glucose can serve as glucosyl donors in eukaryotic microorganisms and plants (10, 33).

Prevotella bryantii B_14 (3), which was formerly classified as *Bacteroides ruminicola* (4) and then *Prevotella ruminicola* (32), is one of the most common bacteria found in the rumen, accounting for up to 19% of total bacterial counts (5). Previous work showed that *P. bryantii* accumulated large amounts of intracellular polysaccharide granules, which disappeared several hours after the culture entered the stationary phase (7, 16). Russell (28, 29) reported that *P. bryantii* accumulated polysaccharide under nitrogen-limiting and glucose-excess conditions in batch as well as in continuous culture. More recently, this polysaccharide was characterized as high-molecular-weight glycogen (21), and glycogen synthesis of this material in *P. bryantii* was regulated by the growth rate as well as the growth substrate. Since as much as 40% of maltose provided to *P. bryantii* was converted into glycogen during cell growth rather than fermentation acids or microbial protein, glycogen synthesis may have important implications in bacterial survival and ruminant nutrition. Therefore, this study investigated the pathway of glycogen biosynthesis in *P. bryantii.*

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MATERIALS AND METHODS

Bacterium and growth conditions. *P. bryantii* B_14 was obtained from J. B. Russell, Cornell University, Ithaca, N.Y. The bacterium was cultured anaerobically at 39°C in a semidefined medium as described previously (20).

Glycogen isolation. Glycogen was isolated by a method previously described (12), with some modifications. Cells were harvested by centrifugation (15,000 × *g* for 10 min at 4°C), washed twice with 0.9% NaCl, and resuspended in 3 ml of 50% KOH (wt/vol). Washed-cell preparations were boiled for 3 h and centrifuged (15,000 × *g* for 10 min at 25°C). Supernatants were collected, and polysaccharide was precipitated by adding 2.8 volumes of cold (4°C) 95% ethanol. After centrifugation (15,000 × *g* for 15 min at 4°C), the pellets were washed twice with cold 70% ethanol and dried under vacuum. The isolated polysaccharide was dissolved in water and desalted by the addition of mixed bed resin (TMD-8; Sigma) until the pH of the solution decreased to approximately 6.5. In preliminary experiments, more than 95% of a commercial glycogen prepared from bovine liver (Sigma G-0885) was recovered after treatment in this manner.

Enzyme assays. Cells were harvested during late-logarithmic growth by centrifugation (15,000 × *g* for 8 min at 4°C), washed twice, and resuspended with 50 mM Tris-HCl (pH 7.2). Crude extracts were prepared by passing the washed cells through a French pressure cell (1,120 kg/cm^2) twice, and cellular debris was removed by centrifugation (32,000 × *g* for 15 min at 4°C). Permeabilized cells were prepared by adding 25 μl of a toluene-ethanol mixture (1:9 [vol/vol]) to 1 ml of cell suspension (approximately 3 mg of protein) as described previously (34). Permeabilized cells were then pelleted by centrifugation (15,000 × *g* for 8 min at 4°C) and resuspended in 50 mM of Tris-HCl (pH 7.2).

NDP-glucose (i.e., ADP-glucose, GDP-glucose, or UDP-glucose) pyrophosphorylase activities were determined spectrophotometrically by monitoring NADPH formation at 340 nm. The reaction mixture (350 μl) contained 100 mM Tris-HCl (pH 7.2), 2.5 mM $MgCl_2$, 2 mM fructose-1,6-bis phosphate, 10 mM sodium fluoride, 5 mM tetrasodium pyrophosphate, 1.5 U of phosphoglucomutase, 2 U of glucose-6-phosphate dehydrogenase, and 5 μl of crude extract (approximately 20 μg of protein). The reaction was started by addition of 2 mM (final concentration) of ADP-glucose, UDP-glucose, or GDP-glucose. The concentration of $MgCl_2$ was less than 3 mM to avoid precipitation with tetrasodium pyrophosphate. All assays were conducted at 39°C.

Glycogen synthase activity was determined by the incorporation of glucosyl units from UDP-[^{3}H]glucose into glycogen. The reaction mixture (600 μl) included 50 mM Tris-HCl (pH 7.2), 3 mM $MgCl_2$, 6 mM glucose-6-phosphate, 3.3 mM UDP-glucose, 10 μl of UDP-[^{3}H]glucose (0.067 mCi/ml; 15.3 Ci/mmol), and permeabilized cells (1 mg of protein). The reaction mixture was incubated at 39°C, and aliquots (100 μl) were withdrawn and immediately added to tubes containing 300 μl of 67.5% KOH to stop the reaction. Glycogen was isolated from this mixture by the ethanol precipitation procedure described above. Dried

glycogen was dissolved in 150 μl of H_2O, and radioactivity was determined by liquid scintillation.

UDP-glucose formation. The reaction mixtures (500 μl) contained 10 μl of [^{14}C]glucose-1-phosphate (50 μCi/ml and 300 μCi/μmol); 5 mM ATP, UTP, or GDP; 5 mM $MgCl_2$; 2 mM fructose-1,6-bis phosphate; 5 mM glucose-1-phosphate; and 50 mM Tris-HCl (pH 7.2). Since previous work (2) indicated that glucose-6-phosphate was required for glycogen synthase activities, preliminary experiments included 10 mM glucose-6-phosphate. However, it was later determined that glucose-6-phosphate was not required for UDP-glucose formation, and the compound was deleted from the mixture. The reaction was initiated by adding permeabilized cells (0.9 mg of protein). Aliquots (100 μl) were withdrawn and immediately boiled in a microcentrifuge tube for 3 min. After centrifugation (13,000 × *g* for 3 min at 25°C), supernatants were transferred into a new tube. The NDP-glucose was separated and identified by thin-layer and paper chromatographies as described below.

Thin-layer and paper chromatographies. Isolated radiolabelled polysaccharide (75 μg) was digested with amyloglucosidase (15 U; Sigma A 3042) at 55°C in sodium acetate (100 mM; pH 4.5) for 2 h, and sugar residues were separated on thin-layer chromatography plates (LK6DF Silica 60 Å; Whatman) by developing in ethyl acetate-water-methanol (8:3:4) for 90 min. Sugars were visualized by spraying with 50% H_2SO_4 and incubating at 120°C for 20 min. After cooling, the bands were carefully scraped from the plate into vials and 3 ml of scintillation fluid was added. UDP-glucose, ADP-glucose, GDP-glucose, and glucose-1-phosphate were separated on thin-layer chromatography plates (LK6DF Silica 60 Å; Whatman) by developing in 95% ethanol–1 M ammonium acetate (5:2; pH 7.5) for 3 h. NDP-glucose appeared as dark bands under UV light (366 nm). The radioactivity in each band was determined after the bands were scraped into vials containing 3 ml of scintillation fluid. NDP-glucoses were also separated by descending paper chromatography. In these assays, samples were spotted onto strips of Whatman no. 1 filter paper (1 by 35 cm) and were separated with 95% ethanol–1 M ammonium acetate (5:2; pH 7.5) for 3 h.

Analyses. The amount of total carbohydrate was measured by an anthrone method (4). The amount of glucose was measured by an enzymatic method with hexokinase and glucose-6-phosphate dehydrogenase (30). The amounts of glycogen and maltose were specifically determined by measuring glucose after incubation with either amyloglucosidase (11) or 5 U of α-glucosidase in 50 mM sodium acetate (pH 6.0), respectively. The amount of protein was determined by the Lowry method (22) after the cells were boiled in 0.2 M NaOH for 15 min.

Materials. [^{14}C]glucose-1-phosphate was purchased from American Radiolabeled Chemicals. [^{14}C]maltose was obtained from Moravek Biochemicals, Inc. UDP-[^{3}H]glucose and all other chemicals and commercial enzymes were obtained from Sigma.

Statistics. All experiments were performed with at least two different cultures, and duplicate determinations were used for the various assays.

RESULTS

NDP-glucose pyrophosphorylase activities. Previous experiments showed that glucose-1-phosphate is a precursor for glycogen synthesis in *P. bryantii* (21). Since ADP-glucose serves as the intermediate in other bacterial glycogen syntheses (24, 25), it seemed reasonable to expect that *P. bryantii* would use a similar pathway. Because the NDP-glucose pyrophosphorylase reaction is reversible, enzyme activities were determined by measuring glucose-1-phosphate formation in the pyrophosphorolysis direction. ADP-glucose- or GDP-glucose-dependent pyrophosphorylase activity was not detected in crude extracts (Fig. 1a). However, when UDP-glucose was added, high activities were observed. When pyrophosphate was omitted from the reaction, there was no detectable activity, indicating that activity was pyrophosphate dependent (Fig. 1b).

UDP-glucose formation. Because the UDP-glucose pyrophosphorylase assay was conducted under conditions which favored glucose-1-phosphate formation, it was necessary to demonstrate UDP-glucose formation from glucose-1-phosphate, since this would be the in vivo direction used during glycogen synthesis. UDP-[^{14}C]glucose was formed from [^{14}C]glucose-1-phosphate by permeabilized cells as identified by thin-layer chromatography (Fig. 2) and paper-descending chromatography (data not shown). Similar results were obtained with crude extracts (data not shown). Virtually no ADP-glucose nor GDP-glucose was detected by either thin-layer or paper chromatography. These results were consistent with the enzyme-linked spectrophotometric assays.

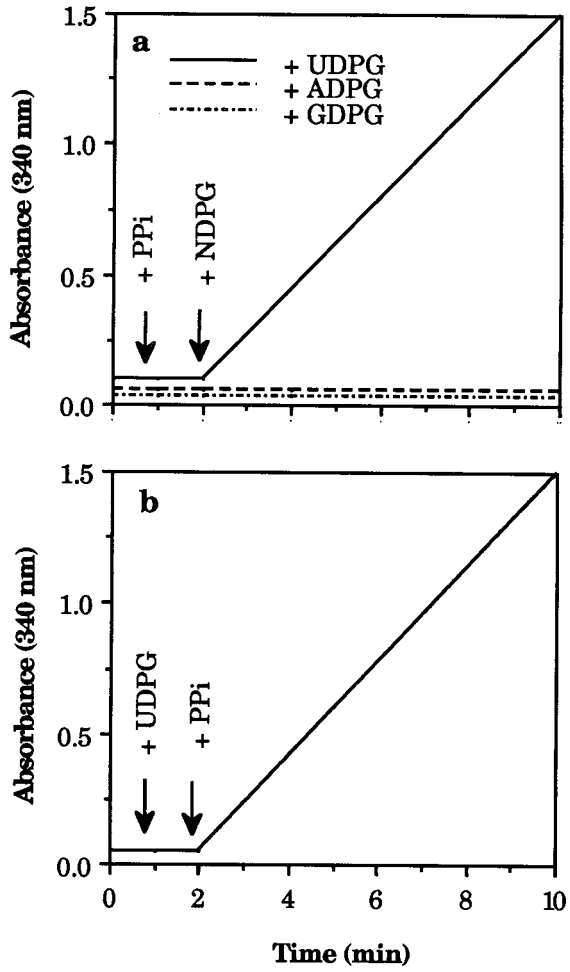

FIG. 1. UDP-glucose (a)- and pyrophosphate (b)-dependent pyrophosphorylase activities. The NDP-glucose pyrophosphorylase assay was conducted in the phosphorolysis direction, and the formation of glucose-1-phosphate was determined spectrophotometrically by monitoring NADPH formation at 340 nm. UDPG, UDP-glucose; ADPG, ADP-glucose; GDPG, GDP-glucose; PPi, pyrophosphate.

Glucosyl unit incorporation from UDP-glucose into glycogen. Although UDP-glucose was synthesized from glucose-1-phosphate by *P. bryantii*, it was still unclear whether UDP-glucose was an intermediate for glycogen biosynthesis. When UDP-[^{3}H]glucose was incubated with permeabilized cells, radioactivity appeared in the polysaccharide extracted by the KOH-ethanol method (Fig. 3). To demonstrate that the radiolabelled material was glycogen, the material was fully digested by amyloglucosidase and the digestion products were separated by thin-layer chromatography (Fig. 4a). Nearly all (>95%) of the radioactivity in the isolated polysaccharide was recovered in the glucose band after enzymatic digestion (Fig. 4b), confirming that the isolated polysaccharide was glycogen. These results demonstrated that glucosyl units from UDP-[^{3}H]glucose were incorporated into glycogen. In parallel experiments with permeabilized cells or growing cells, the radioactivity from either [^{14}C]glucose-1-phosphate or [^{14}C]maltose, respectively, also appeared in glycogen (data not shown).

Since radiolabelled ADP-glucose is not commercially available, it was not possible to directly test whether this compound served as a precursor of glycogen synthesis. Therefore, competition studies were designed to evaluate whether ADP-glu-

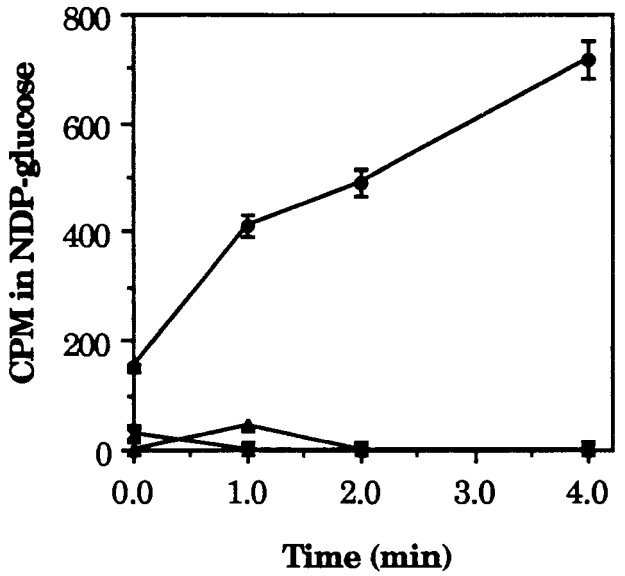

FIG. 2. UDP-[^{14}C]glucose formation from [^{14}C]glucose-1-phosphate by permeabilized cells. The reaction mixture contained [^{14}C]glucose-1-phosphate; ATP, UTP, or GTP; $MgCl_2$; fructose-1,6-bis phosphate; glucose-1-phosphate; Tris (pH 7.2); and permeabilized cells. Aliquots (100 μl) were taken at various times, NDP-glucoses were separated by thin-layer chromatography, and radioactivity was measured as described in Materials and Methods. ●, UDP-glucose; ■, ADP-glucose; ▲, GDP-glucose; vertical bars, standard errors of the means.

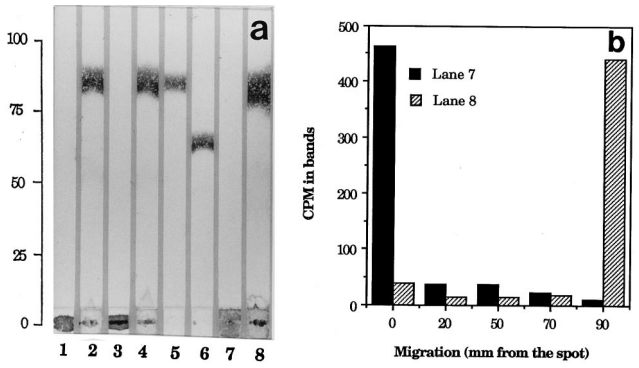

FIG. 4. Amyloglucosidase digestion of radiolabelled polysaccharide. (a) The isolated radiolabelled polysaccharide synthesized from UDP-[^{3}H]glucose (see Fig. 3) was digested with amyloglucosidase, and the digestion suspension was separated by thin-layer chromatography (vertical axis represents millimeters). Lanes 1, 3, and 7, commercial glycogen, unlabelled *P. bryantii* glycogen, and isolated radiolabelled material, respectively; lanes 2, 4, and 8, enzymatic digestion of samples in lanes 1, 3, and 7, respectively; lanes 5 and 6, glucose and maltose standards, respectively. (b) Nearly all radioactivity in isolated material migrated with the glucose band.

cose was involved in glycogen formation. When a 20-fold excess of unlabelled ADP-glucose (66 mM) was added to a reaction mixture containing UDP-[^{3}H]glucose, there was little effect on incorporation of the radiolabel into glycogen (Fig. 5). In contrast, when a fourfold excess of unlabelled UDP-glucose (13 mM) was added, incorporation was reduced by 75%. In a similar experiment, incorporation of glucose into glycogen from [^{14}C]glucose-1-phosphate was monitored, and the addition of unlabelled UDP-glucose again dramatically reduced [^{14}C]glucosyl incorporation while no decrease of [^{14}C]glucosyl incorporation was observed when unlabelled ADP-glucose was added (Fig. 5). These results strongly suggested that ADP-glucose was not incorporated into glycogen.

Enzyme activities in cells grown in different substrates. Previous experiments showed that there was considerable variation in glycogen accumulation when cells were grown on different substrates (21); therefore, enzymes involved in glycogen synthesis were measured in cells grown on these different substrates. Both UDP-glucose pyrophosphorylase and glycogen synthase activities were detected in all cultures, but their activities were substrate dependent (Table 1). UDP-glucose pyrophosphorylase activities in maltose- and cellobiose-grown cultures were higher than those in sucrose- and glucose-grown cultures. Glycogen synthase activities were more dramatically

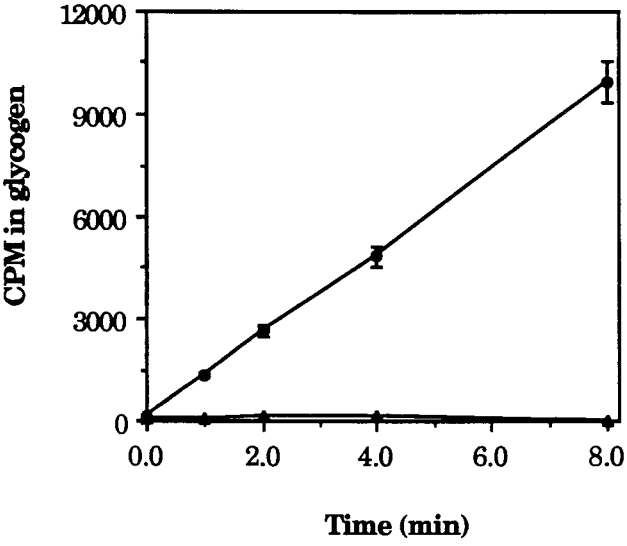

FIG. 3. Glycogen synthesis from UDP-[^{3}H]glucose by permeabilized cells (●) or boiled cells (▲). The reaction mixture contained Tris buffer (pH 7.2), $MgCl_2$, UDP-[^{3}H]glucose, and permeabilized cells. Aliquots were withdrawn from the reaction mixture, glycogen was isolated, and radioactivity was determined. Vertical bars, standard errors of the means.

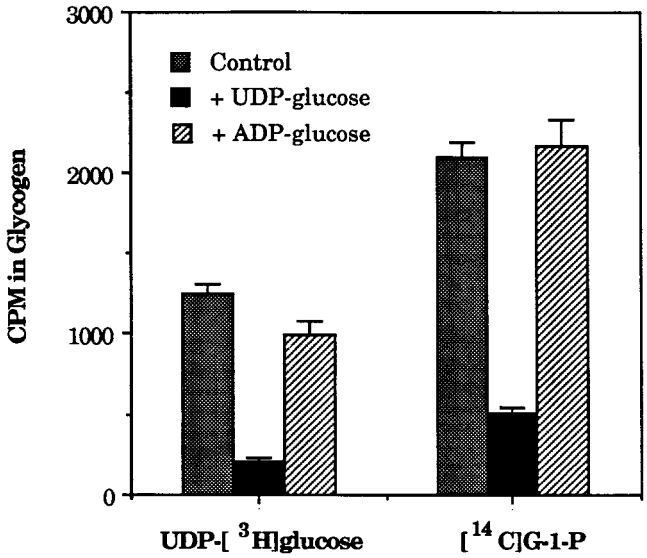

FIG. 5. Effects of adding unlabelled UDP-glucose (13 mM) or ADP-glucose (66 mM) on glucose incorporation into glycogen from UDP-[^{3}H]glucose (3.3 mM) or [^{14}C]glucose-1-phosphate (3.3 mM). The control contained only radiolabelled substrate. Vertical bars, standard errors of the means.

TABLE 1. Effects of growth substrates on enzyme activities in *P. bryantii*

Growth substrate[a]	Growth rate (h^{-1})	Sp act (nmol/min/mg of protein)	
		UDP-glucose pyrophosphorylase[b]	Glycogen synthase
Maltose	0.46 ± 0.02	2,366 ± 61	48 ± 8
Cellobiose	0.67 ± 0.03	2,043 ± 45	20 ± 6
Sucrose	0.73 ± 0.03	1,492 ± 57	11 ± 4
Glucose	0.90 ± 0.04	1,548 ± 39	8 ± 2

[a] Cells were grown in 4 g of carbohydrate per liter. Values are averages of two experiments ± standard errors.
[b] Activity was measured in the pyrophosphorolysis direction.

affected by growth substrates, with the lowest activity observed in glucose-grown cells. In general, there was an inverse relationship between growth rate and enzyme activities.

Allosteric regulation. UDP-glucose pyrophosphorylase activity required fructose-1,6-bis phosphate, and pyruvate could not substitute for this requirement (Fig. 6). The concentration of fructose-1,6-bis phosphate required for 50% of maximal activity was 240 μM. In eukaryotic cells, glucose-6-phosphate is an important activator of glycogen synthase (2); however, glucose-6-phosphate did not stimulate activity in *P. bryantii* when it was provided at concentrations of as high as 10 mM (data not shown).

DISCUSSION

Glycogen biosynthesis in all bacteria studied thus far involves ADP-glucose as an intermediate; ADP-glucose is synthesized from glucose-1-phosphate and ATP by ADP-glucose pyrophosphorylase (24–26). Previous experiments with *P. bryantii* B_14 demonstrated that glucose-1-phosphate apparently was the precursor of glycogen formation (21), and it was reasonable to hypothesize that ADP-glucose was the next intermediate in glycogen biosynthesis. It was, thus, surprising to find that *P. bryantii* did not possess ADP-glucose pyrophosphorylase but did have UDP-glucose-dependent pyrophosphorylase activity (Fig. 1). The following evidence clearly demonstrated that glycogen biosynthesis by *P. bryantii* was via UDP-glucose: (i) radiolabelled UDP-glucose (but not ADP-glucose or GDP-glucose) was synthesized from [^{14}C]glucose-1-phosphate (Fig. 2); (ii) radiolabelled glucosyl units from UDP-[^{3}H]glucose were incorporated into glycogen (Fig. 3); and (iii) addition of ADP-glucose did not affect glycogen synthesis from either UDP-glucose or glucose-1-phosphate (Fig. 5). To our knowledge, all bacteria which have been investigated thus far only use ADP-glucose as an intermediate for glycogen synthesis. The results with *P. bryantii* are the first report of a bacterium exclusively using UDP-glucose as the glucosyl donor to synthesize glycogen.

Either UDP-glucose or ADP-glucose can serve as the glucosyl donors for glycogen biosynthesis in eukaryotic cells (13, 19). In *Neurospora crassa*, UDP-glucose is the most active glucosyl donor, but ADP-glucose can partially substitute for UDP-glucose (10). Although ADP-glucose is the major glucosyl donor for starch synthesis in plants, UDP-glucose also participates in polysaccharide formation (33). UDP-glucose is the predominant precursor for glycogen synthesis in mammalian cells (2). UDP-glucose pyrophosphorylases were found in several bacterial species (35), but the enzymes are not involved in glycogen biosynthesis (15, 17). The genes encoding UDP-glucose pyrophosphorylase in mammalian cells show little similarity to their bacterial counterparts (18, 23). The reasons why *P. bryantii* lacks ADP-glucose pyrophosphorylase activity and why its glycogen synthase is specific for UDP-glucose are not clear, but it is possible that other eubacterial species possess these characteristics, and this deserves further study.

Bacterial glycogen biosynthesis is subject to both allosteric and genetic regulation (24, 25). Fructose-1,6-bis phosphate is an activator for glycogen synthesis in a number of bacterial species including *E. coli*, *Rhodopseudomonas sphaeroides*, and *Agrobacterium tumefaciens* (24). The stimulation by fructose-1,6-bis phosphate of UDP-glucose pyrophosphorylase activity in *P. bryantii* suggested that fructose-1,6-diphosphate may play an important role in controlling carbon flow to glycogen synthesis versus glycolysis. Such regulation would make sense, since fructose-1,6-bis phosphate is a glycolytic intermediate and also since UDP-glucose synthesis is the first step leading to glycogen synthesis from glucose-1-phosphate. The half-maximal activation concentration for fructose-1,6-bis phosphate in *P. bryantii* (240 μM) was higher than the value in *E. coli* (120 μM [14]). Dietzler et al. reported that there was a positive relationship between the rate of glycogen synthesis in *E. coli* and the accompanying intracellular level of fructose-1,6-bis phosphate (9), suggesting that the allosteric regulation of glycogen biosynthesis may function in vivo.

The observation that glycogen synthetic enzyme production was affected by the growth substrate suggested that there may also be genetic regulation of glycogen synthesis. The sixfold variation in glycogen synthase activities of cells grown on different substrates may at least partially explain the various amounts of glycogen found in previous experiments (21). In eukaryotic cells, glycogen synthase uses UDP-glucose as the glucose donor and is the major rate-limiting step for glycogen synthesis (1). Glycogen synthase is subject to covalent modification (i.e., phosphorylation and dephosphorylation) and allosteric regulation by a number of effector molecules (6, 27). The dephosphorylated form is active, whereas the phosphorylated enzyme requires glucose-6-phosphate for maximal activity. Although glycogen synthase in *P. bryantii* used UDP-glucose as the substrate, glucose-6-phosphate had no allosteric effect on enzyme activity. The branching enzyme is necessary to form the mature glycogen molecule, and such activity needs to be identified for *P. bryantii.*

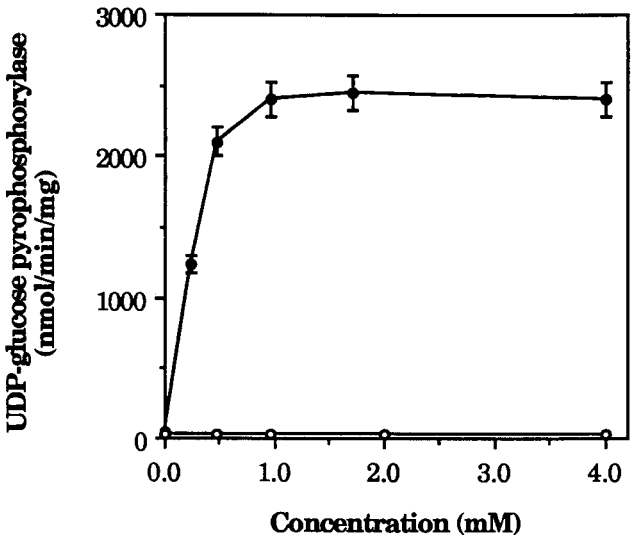

FIG. 6. Regulation of UDP-glucose pyrophosphorylase activity in the presence of fructose-1,6-bis phosphate (●) or pyruvate (○). The enzyme assay was described in Materials and Methods. Vertical bars, standard errors of the means.

There was an inverse relationship between the growth rate of *P. bryantii* and glycogen synthesis (21) as well as the synthesis of glycogen enzymes (Table 1). However, it is unknown whether this growth rate-dependent response involved allosteric effects on the pyrophosphorylase mediated by intracellular fructose-1,6-bis phosphate levels, substrate-dependent regulation of glycogen synthase gene expression, or a combination of both of these possibilities. The situation is further confounded by the fact that intracellular glucose-1-phosphate levels were substrate dependent; maltose- and cellobiose-grown cells had concentrations of 1.9 and 0.3 mM, respectively, but glucose-1-phosphate was not detected in either glucose- or sucrose-grown cultures (21). The presence of maltose and cellobiose phosphorylases, which lead to the formation of glucose-1-phosphate, supported the contention that glucose-1-phosphate levels were important in determining the extent of glycogen accumulation (21). Further study of enzyme activities and intracellular metabolite levels in continuous cultures grown at different growth rates should provide more information on regulatory control mechanisms.

P. bryantii is a major amylolytic microorganism in the rumen and produces high-level amylase activity which degrades starch, an important component of many ruminant diets (8). Rapid starch fermentation often causes the digestive disorder lactic acidosis. Some rumen protozoa are known to sequester starch granules in specialized internal organelles and prevent the rapid fermentation associated with acidosis (36). Because *P. bryantii* can convert maltose into intracellular glycogen and since this organism is resistant to low pH values that are typical of lactic acidosis (31), glycogen accumulation may have important implications in influencing the starch fermentation rate and preventing the detrimental effects of lactic acidosis. Since energy sources are often transiently available in the rumen, mechanisms of survival under starvation situations become important for ruminal bacteria. The fact that glycogen in *P. bryantii* is fermented into acetate and succinate during the stationary phase indicates that the polysaccharide may function as an energy reserve (21). However, the exact physiological and ecological significance of glycogen accumulation is uncertain. Elucidation of the glycogen biosynthesis pathway provides information to further investigate the role of glycogen in rumen function.

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