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Pathway and Sites for Energy Conservation in the Metabolism of Glucose by *Selenomonas ruminantium*

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On the basis of enzyme activities detected in extracts of *Selenomonas ruminantium* HD4 grown in glucose-limited continuous culture, at a slow (0.11 h^{-1}) and a fast (0.52 h^{-1}) dilution rate, a pathway of glucose catabolism to lactate, acetate, succinate, and propionate was constructed. Glucose was catabolized to phosphoenol pyruvate (PEP) via the Emden-Meyerhoff-Parnas pathway. PEP was converted to either pyruvate (via pyruvate kinase) or oxalacetate (via PEP carboxykinase). Pyruvate was reduced to L-lactate via a NAD-dependent lactate dehydrogenase or oxidatively decarboxylated to acetyl coenzyme A (acetyl-CoA) and CO_2 by pyruvate:ferredoxin oxidoreductase. Acetyl-CoA was apparently converted in a single enzymatic step to acetate and CoA, with concomitant formation of 1 molecule of ATP; since acetyl-phosphate was not an intermediate, the enzyme catalyzing this reaction was identified as acetate thiokinase. Oxalacetate was converted to succinate via the activities of malate dehydrogenase, fumarase and a membrane-bound fumarate reductase. Succinate was then excreted or decarboxylated to propionate via a membrane-bound methylmalonyl-CoA decarboxylase. Pyruvate kinase was inhibited by P_i and activated by fructose 1,6-bisphosphate. PEP carboxykinase activity was found to be 0.054 μmol min^{-1} mg of protein^{-1} at a dilution rate of 0.11 h^{-1} but could not be detected in extracts of cells grown at a dilution rate of 0.52 h^{-1}. Several potential sites for energy conservation exist in *S. ruminantium* HD4, including pyruvate kinase, acetate thiokinase, PEP carboxykinase, fumarate reductase, and methylmalonyl-CoA decarboxylase. Possession of these five sites for energy conservation may explain the high yields reported here (56 to 78 mg of cells [dry weight] mol of glucose^{-1}) for *S. ruminantium* HD4 grown in glucose-limited continuous culture.

Selenomonas ruminantium HD4 is a gram-negative, anaerobic bacterium that ferments glucose to lactate, acetate, propionate, and CO_2 (1). The amounts and proportions of the products formed depend on growth conditions. In batch culture, with glucose as the carbon source, the cells first produce D,L-lactate and then ferment the lactate to acetate and propionate (22, 33). Continuous-culture studies in glucose-limited chemostats revealed a shift from an acetate, propionate, and succinate fermentation at slow dilution rates to a mostly lactate fermentation at fast rates (22, 27, 33).

Very little was known about how *S. ruminantium* ferments glucose to the various products listed above. In a limited survey of enzymatic activities, using cells grown in batch culture on glucose, several enzymes were detected. These included aldolase, NAD-dependent lactate dehydrogenase (LDH), fumarate reductase, and an NADP-dependent malic enzyme (15). Enzyme activities assayed but not detected were phosphotransacetylase, acetate kinase, and malate dehydrogenase. A lactic acid racemase was recently found in cells grown in glucose-limited continuous culture (22).

In addition to studies determining specific enzyme activities, labeling studies were also done to learn how propionate is formed from lactate. Paynter and Elsden (26) grew *S. ruminantium* HD4 on [2-^{14}C]D,L-lactate and demonstrated that the propionate produced contains label distributed equally (i.e., randomized) between the C-2 and C-3 positions. They concluded that the fermentation of lactate to propionate proceeds via a nonisomeric intermediate, succinate. It seems possible that succinate is also an intermediate when glucose is fermented to propionate.

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The present study was undertaken to determine the complete pathway used by *S. ruminantium* HD4 to ferment glucose to all the products mentioned above. Additionally, the potential sites for energy conservation were determined so that a clearer understanding might be gained of how this organism is able to achieve the extremely high cell yields (62 to 98 g of cells [dry weight] mol of glucose^{-1}) reported in this study and by others (12, 27, 29). The mechanism used by this organism to regulate product formation at different dilution rates is described elsewhere (23).

MATERIALS AND METHODS

S. ruminantium HD4 subsp. *lactilytica* was obtained from M. P. Bryant (1) and was cultured anaerobically at 39°C on modified medium 10, based on the medium 10 of Caldwell and Bryant (2), as described elsewhere (22). Continuous culture experiments were performed in a bench top chemostat (model C-30; New Brunswick Scientific Co., Edison, N.J.) (28) with a 379-ml working volume and contained modified medium 10 with 2.78 mM glucose. Steady-state conditions were assumed after at least four volume turnovers had occurred. Cell density in the chemostat was monitored by using a Cary 219 spectrophotometer (Varian Associates, Palo Alto, Calif.). At each time point in the experiment depicted in Fig. 1, a minimum of two samples were removed from the chemostat and analyzed for levels of glucose, acetate, propionate, succinate, and L- and D-lactate (22), as well as cell dry weight (14).

Enzyme assays. Cells grown in continuous culture were harvested and disrupted by passage through a French pressure cell at 20,000 lb/in^2. Soluble and membrane fractions were prepared as described previously (22). Assay conditions and references for the nonradioactive enzyme assays

TABLE 1. Conditions for nonradioactive assays of enzyme activities[a]

Enzyme (reference)	Assay conditions
NAD-LDH	70 mM KPO_4, pH 6.2, 5 mM sodium pyruvate, 0.2 mM NADH
Lactate racemase (4)	100 mM sodium acetate, pH 5.0, 20 mM L- or D-lactate; time course; assay for the appearance of the other isomer
Aldolase	50 mM Tris hydrochloride, pH 7.5, 80 mM KCl, 5 mM PEP, 17 U triose phosphate isomerase, 5 U α-glycerophosphate dehydrogenase, 1.5 mM FBP
Pyruvate kinase (3)	50 mM TEA, pH 7.5, 8 mM $MgCl_2$, 80 mM KCl, 5 mM PEP, 5 mM ADP, 2 mM FBP, 10 U LDH, 0.2 mM NADH
Pyruvate:ferredoxin oxidoreductase	50 mM KPO_4, pH 7.0, 50 mM sodium pyruvate, 0.2 mM CoA, 25 mM mercaptoethanol 1 mM benzyl viologen; anaerobic assay conditions
Acetate thiokinase	50 mM KPO_4, pH 7.0, 0.5 mM acetyl-CoA, 2 mM $MgCl_2$, 1 mM ADP, 1 mM EDTA, 1 mM α-ketoglutarate, 50 mU α-ketoglutarate dehydrogenase, 0.4 mM APAD
Phosphotransacetylase (35)	50 mM KPO_4, pH 7.2, 2.5 mM acetyl-CoA; time course; assay for acetyl-P formation
Phosphotransacetylase (16)	50 mM TEA, pH 7.4, 2 mM acetyl-P, 1 mM CoA, 2 mM GSH, 20 mM $(NH_4)_2SO_4$; formation of acetyl-CoA thioester bond
PEP carboxykinase (32)	50 mM succinate-borate, pH 5.5, 10 mM $MgCl_2$, 10 mM ADP, 25 mM $NaHCO_3$, 20 mM PEP, 2 U MDH, 0.15 mM NADH
PEP carboxylase (21)	66 mM Tris hydrochloride, pH 9.0, 10 mM $MgCl_2$, 5 mM PEP, 10 mM $NaHCO_3$, 2 U MDH, 0.15 mM NADH
PEP carboxytransphosphorylase (42)	10 mM KPO_4, pH 6.8, 2 mM PEP, 10 mM $MgCl_2$, 30 mM $NaHCO_3$, 0.1 mM $CoCl_2$, 2 U MDH, 0.15 mM NADH
Malate dehydrogenase	50 mM Tris hydrochloride, pH 9.0, 1 mM NAD^+, 5 mM L-malate
Fumarate reductase (18)	100 mM KPO_4, pH 7.2, 5 mM fumarate, 0.15 mM NADH; NADH-dependent reduction of fumarate
Methylmalonyl-CoA decarboxylase (10)	10 mM $NaAsO_4$, pH 7.0, 0.15 mM R,S-methylmalonyl-CoA, 50 mM KCl, 20 U phosphotransacetylase; AsO_4-dependent hydrolysis of the thioester bond of propionyl-CoA
Pyruvate carboxylase (34)	100 mM Tris hydrochloride, pH 7.8, 10 mM sodium pyruvate, 2 mM ATP, 5 mM $MgCl_2$, 15 mM $NaHCO_3$, 0.2 mM acetyl-CoA; time course; assay for OAA with MDH and NADH
OAA decarboxylase (5)	100 mM Tris hydrochloride, pH 7.8, 20 mM NaCl, 1 mM OAA; reduction in A_{265} of OAA
OAA transcarboxylase (43)	350 mM KPO_4, pH 6.8, 10 mM sodium pyruvate, 0.4 mM methylmalonyl-CoA, 10 mM $MgCl_2$; time course; assay for OAA with MDH and NADH
NADP-dependent malic enzyme (31)	100 mM Tris hydrochloride, pH 7.5, 5 mM L-malate, 1 mM $MnCl_2$, 0.5 mM $NADP^+$
NAD-dependent malic enzyme (30)	50 mM MOPS, pH 7.0, 2 mM L-malate, 1 mM $MnCl_2$, 1 mM NAD^+
Malate synthase	50 mM Tris hydrochloride, pH 7.5, 10 mM $MgCl_2$, 0.5 mM acetyl-CoA, 5 mM $MnCl_2$, 2 mM sodium glyoxylate, 0.5 U malic enzyme, 1 mM $NADP^+$

[a] KPO_4, Potassium phosphate; TEA, triethanolamine; APAD, acetyl-pyridine adenine dinucleotide; GSH, glutathione; MDH, malate dehydrogenase; MOPS, morpholinepropanesulfonic acid.

are listed in Table 1. All absorbance readings were made in a Cary 219 spectrophotometer by using cuvettes with a 1-cm light path.

Radioactive $^{14}CO_2$ fixation assays were based on a modified version of the method of Steinmüller and Böck (36). The total volume of each assay mixture was 0.4 ml. Each assay mixture, prepared in a 1.5-ml microcentrifuge tube, was incubated at 39°C. The reaction was started by the addition of the cell extract. At 0, 1, 2, 5, 10, and 20 min, 50 μl of the reaction mixture was pipetted into a liquid scintillation vial containing 0.3 ml of 3 M $HClO_4$. The vials were then incubated at 60°C for 20 min to drive off unfixed $^{14}CO_2$. After the vials were cooled to room temperature, liquid scintillation cocktail was added, and the radioactivity remaining was counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.). The assay mixture for phosphoenolpyruvate (PEP) carboxylase contained 100 mM Tris hydrochloride, pH 9.0, 20 mM PEP, 10 mM $MgCl_2$, 5 mM fructose-1,6-bisphosphate (FBP), 5 mM fructose-6-phosphate, 1 mM acetyl coenzyme A (acetyl-CoA), 25 mM $NaH^{14}CO_3$ (0.1μCi/μmol), and 0.3 mg of protein. The assay mixture for pyruvate carboxylase contained 50 mM Tris hydrochloride, pH 7.5, 10 mM sodium pyruvate, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 5 mM ATP, 25 mM $NaH^{14}CO_3$ (0.1μCi/μmol), and 0.3 mg of protein. The PEP carboxytransphosphorylase assay mixture contained 10 mM $KHPO_4$, pH 6.8, 2 mM PEP, 10 mM $MgCl_2$, 0.1 mM $CoCl_2$, 25 mM $NaH^{14}CO_3$ (0.1μCi/μmol), and 0.3 mg of protein. The NADPH-dependent carboxylation of pyruvate by malic enzyme was assayed under the following conditions: 50 mM Tris hydrochloride, pH 7.5, 5 mM sodium pyruvate, 1 mM NADPH, 25 mM $NaH^{14}CO_3$ (0.1μCi/μmol), and 0.3 mg of protein.

Protein was determined by the method of Hartree (8). Biochemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

RESULTS

The fermentation products produced by *S. ruminantium* HD4 in glucose-limited continuous culture are shown in Fig. 1B. The pH was maintained by the buffering capacity of the medium at an average of 6.8, and it never varied by more than 0.1 U. Glucose was not detected at any dilution rate, and carbon balances ranged from 98 to 112% carbon recovered at the dilution rates tested. At slow dilution rates, acetate and propionate were the major fermentation products formed, while at fast rates, lactate was the major product. This shift in fermentation took place at a dilution rate of roughly 0.2 h^{-1}, which was also the dilution rate at which large amounts of succinate were produced, as previously described (22). Figure 1A shows the molar growth

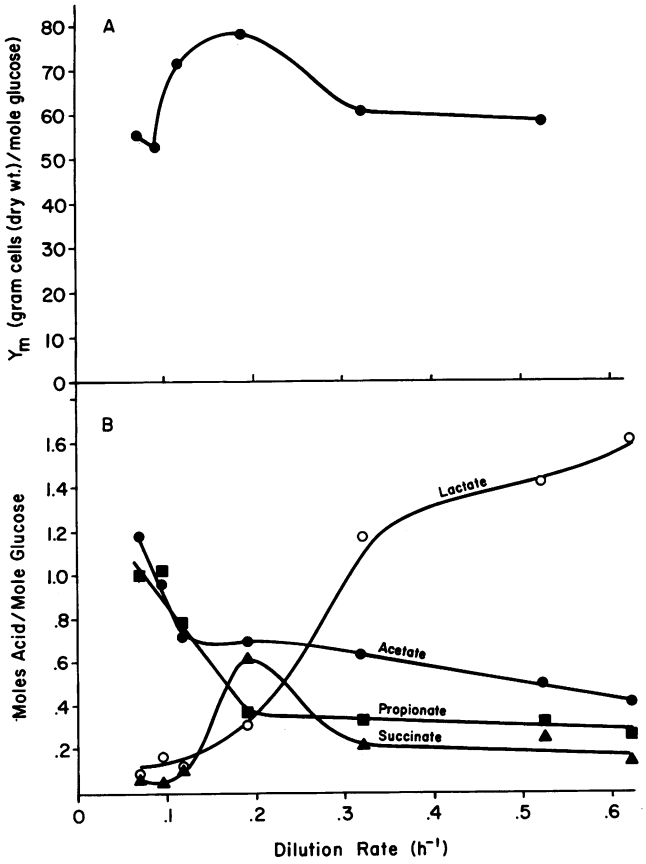

FIG. 1. (A) Molar growth yield of *S. ruminantium* HD4 over the course of the experiment shown in panel B. (B) Pattern of fermentation end products produced by *S. ruminantium* grown in glucose-limited continuous culture. Symbols: ○, lactate; ●, acetate; ■, propionate; ▲, succinate.

yields of *S. ruminantium* HD4 over the course of the experiment in Fig. 1B. The highest yield was observed at a dilution rate of 0.2 h^{-1}.

The fact that the fermentation pattern of *S. ruminantium* changed with the dilution rate suggested that the activity or synthesis of the enzymes involved in glucose catabolism also changed with the dilution rate. To test this hypothesis, the specific activities of enzymes responsible for glucose catabolism were measured in extracts of cells grown at dilution rates of 0.11 and 0.52 h^{-1}. The results are shown in Table 2. With two exceptions, the activities of enzymes did not vary more than twofold between the high and low dilution rates. Lactic acid racemase activity was fourfold greater at the higher dilution rate; this was reflected in the amount of D-lactate formed (22). The PEP carboxykinase, with a specific activity of 0.054 mmol min^{-1} mg of $protein^{-1}$ at a dilution rate of 0.11 h^{-1} was not detected in extracts of cells grown at a dilution rate of 0.52 h^{-1}.

On the basis of the enzyme activities detected in this organism (Table 2), a scheme of the pathway used by *S. ruminantium* HD4 to ferment glucose was constructed (Fig. 2). There are two branch points in this pathway. The first of these involves PEP, the common substrate of both pyruvate kinase and PEP carboxykinase. At the second branch point, pyruvate is the substrate for both LDH and pyruvate: ferredoxin oxidoreductase.

The enzyme that converts PEP to pyruvate in *S. ruminantium* is pyruvate kinase. This enzyme was strongly inhibited by P_i. Only 2.0 mM P_i was required to reduce the activity of the enzyme to 2% of its uninhibited rate. FBP (2.2 mM) restored the activity to normal when 2.0 mM P_i was present. When P_i was absent, FBP activated the enzyme; 4 mM FBP increased the initial velocity by 75%. The pyruvate kinase exhibited a sigmoidal saturation curve with PEP as the limiting substrate. The concentration of PEP that resulted in an activity equal to 0.5 V_{max} ($[S]_{.5}$) was 0.086 mM.

TABLE 2. Specific activities of enzymes in cell extracts of *S. ruminantium* HD4 grown in glucose-limited continuous culture

Enzyme	Sp Act (μmol min^{-1} mg of $protein^{-1}$) at dilution rate (h^{-1}) of:	
	0.11	0.52
NAD-LDH[a]	11.2	6.8
Lactate racemase[a]	0.012	0.041
Pyruvate kinase	1.36	1.00
Pyruvate:ferredoxin oxidoreductase	0.87	1.08
Acetate thiokinase	0.076	0.089
PEP carboxykinase	0.054	ND[b]
Malate dehydrogenase	0.43	0.20
Fumarate reductase[c]	0.036	0.031
Methylmalonyl-CoA decarboxylase[c]	0.049	0.030

[a] Data are from reference 22.
[b] ND, Not detectable.
[c] Specific activity measured as micromoles per minute per milligram of membrane protein (see Materials and Methods).

Lactate branch. The first enzyme in this branch, the NAD-dependent LDH, has been purified, and it exhibits positive cooperativity for the binding of pyruvate (23). It produces L-lactate from pyruvate, which is then converted to D-lactate by a lactate racemase, as reported previously (22).

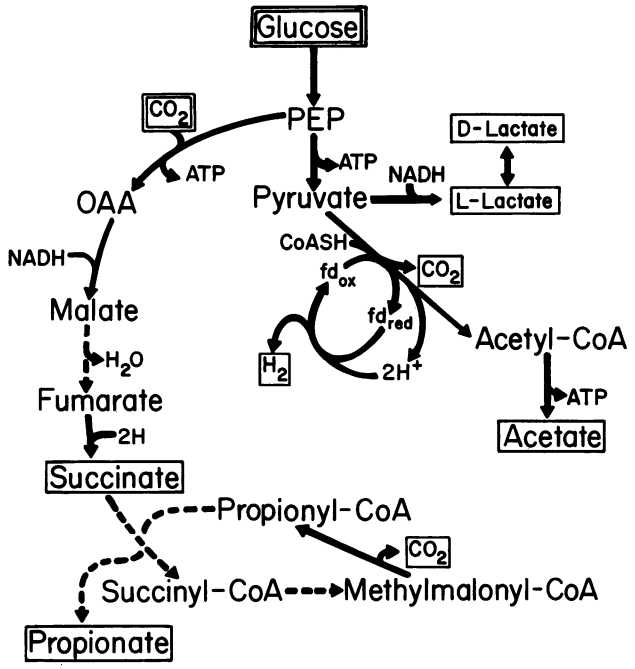

FIG. 2. Pathway of glucose catabolism by *S. ruminantium* HD4. ——, enzyme activities detected in cell extracts; ---, enzymatic reactions assumed to be present. fd_{ox}, Ferredoxin oxidized; fd_{red}, ferredoxin reduced.

Acetate branch. This is the first report of a pyruvate: ferredoxin oxidoreductase, the first enzyme in the acetate branch, in this organism. With pyruvate as the substrate, the enzyme exhibited Michaelis-Menten kinetics and had a K_m for pyruvate of 0.49 mM. The next enzymatic step in acetate production was the enzyme activity identified as acetate thiokinase (Table 2 and Fig. 2). The thiokinase appeared to produce ATP and acetate from acetyl-CoA, ADP, and P_i in a single step:

$$\text{acetyl-CoA} + \text{ADP} + P_i \rightarrow \text{acetate} + \text{ATP} + \text{CoA} \quad (1)$$

In most bacteria, this same reaction is carried out by two enzymes, phosphotransacetylase and acetate kinase. These enzymes utilize acetyl-phosphate (acetyl-P) as a high-energy intermediate:

$$\text{acetyl-CoA} + P_i \leftrightarrow \text{acetyl-P} + \text{CoA} \quad \text{(phosphotransacetylase)} \quad (2)$$

$$\text{acetyl-P} + \text{ADP} \leftrightarrow \text{acetate} + \text{ATP} \quad \text{(acetate kinase)} \quad (3)$$

$$\text{sum: acetyl-CoA} + \text{ADP} + P_i \leftrightarrow \text{acetate} + \text{ATP} + \text{CoA}$$

There were several lines of evidence to support the conclusion that *S. ruminantium* formed acetate and ATP from acetyl-CoA by using a single enzyme (i.e., without acetyl-P being a soluble intermediate in the reaction). Cell extracts catalyzed the formation of free CoA from acetyl-CoA. This reaction required the presence of both P_i and ADP. In a two-enzyme system such a double requirement would reflect the fact that P_i and ADP are substrates in reactions 2 and 3, respectively. None of these reactions could, however, be detected in either direction. Adenosine diphosphate might be an activator of phosphotransacetylase, but a plot of $1/V_0$ versus 1/ADP was linear; the K_m value was 27.5 μM (data not shown). This indicated that ADP was acting as a substrate and not as an activator of the enzyme. As can be seen in Fig. 3, acetyl-P was, however, hydrolyzed by cell extracts but the hydrolysis of acetyl-P was inhibited by ADP, a compound that should act as a substrate for acetate kinase and not as an inhibitor. These results do not completely exclude the possibility that acetyl-P is an intermediate in reaction 1, since it may be present in a bound form on the enzyme.

Propionate branch. PEP carboxykinase is a site for energy conservation in *S. ruminantium* HD4, because of the net synthesis of 1 molecule of ATP per PEP molecule carboxylated. The PEP carboxykinase exhibited a sigmoidal saturation curve with PEP as the substrate and an $[S]_{.5}$ of 5.5 mM (data not shown).

The next enzymes in the propionate branch are malate dehydrogenase and fumarate reductase. The latter enzyme was found to be membrane bound, which is a requirement for proton extrusion to occur. In *S. ruminantium* HD4, the methylmalonyl-CoA decarboxylase was also associated with the membrane fraction. Since complete inactivation of the enzyme occurred if the membrane extracts were preincubated with avidin prior to the assay, biotin probably plays a key role in the activity of the enzyme in *S. ruminantium*.

The absence of PEP carboxykinase activity at the fast dilution rate (0.52 h^{-1}) posed a problem with the pathway as shown in that significant quantities of propionate and succinate were produced (Fig. 1B). Since intermediates in propionate formation (e.g., oxalacetate [OAA] and malate) are normally produced via carboxylation reactions involving PEP or pyruvate, another enzyme, not yet detected, must be functioning to produce a 4-carbon intermediate at fast dilution rates. Cell extracts from continuous culture and batch growth were assayed for enzymes other than PEP carboxykinase that could produce OAA or malate. These included PEP carboxylase, PEP carboxytransphosphorylase, methylmalonyl-CoA:pyruvate transcarboxylase, malic enzyme (NADP and NAD dependent), malate synthase, OAA decarboxylase and pyruvate carboxylase. No activities for any of these enzymes, except pyruvate carboxylase, were detected, and extremely low levels of this enzyme (6×10^{-4} μmol min^{-1} mg of $protein^{-1}$) were found only in batch-grown cells by a sensitive $^{14}CO_2$ fixation assay. It is possible that pyruvate carboxylase is present in cells growing at a fast dilution rate, but it could not be detected by our assay owing to the absence of an unidentified activator. One possible activator might be acetyl-CoA, which has been shown to be a potent activator of pyruvate carboxylases from mammalian and bacterial sources (34). However, the presence of 2 mM acetyl-CoA in the reaction mixture failed to increase the low pyruvate carboxylase activities detected in batch-grown cells. FBP and fructose-6-phosphate, at a concentration of 2 mM each, also failed to increase the pyruvate carboxylase activity.

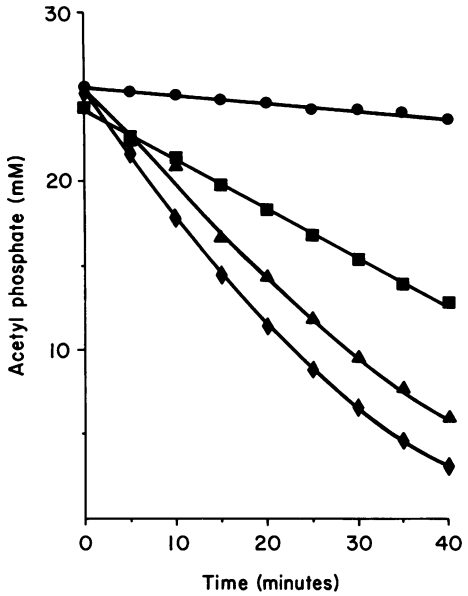

FIG. 3. Hydrolysis of acetyl-P by cell extracts of *S. ruminantium* HD4. The complete reaction mixture contained (in 2 ml) 50 mM triethanolamine, pH 7.0, 25 mM acetyl-P, 10 mM ADP, 25 mM $MgCl_2$, 12.5 mM EDTA, 10 mM KH_2PO_4, and 0.6 mg of cell protein. Symbols: ■, complete reaction mixture; ▲, minus ADP; ◆, minus KH_2PO_4; ●, minus cell extract.

DISCUSSION

From the work reported here, a pathway for glucose metabolism by *S. ruminantium* has emerged for the first time. The presence of aldolase in *S. ruminantium* HD4, reported here and by others (15), indicates that glucose is catabolized initially through the Emden-Meyerhoff-Parnas pathway in this organism. Aldolase is a key enzyme in this pathway of glucose catabolism to PEP (7).

Acetate branch. The production of acetate proceeds via a different series of steps in *S. ruminantium*, when compared with its production in other bacteria. The pyruvate:ferredoxin oxidoreductase first produces acetyl-CoA from pyruvate.

The acetate thiokinase then appears to produce acetate, CoA, and ATP via a single enzyme. Such an activity has been reported previously in facultatively anaerobic protozoa (19, 24). This is the first report of an acetate thiokinase that produces acetate from acetyl-CoA in a bacterium. Other bacterial acetate thiokinases produce acetyl-CoA from acetate, requiring an input of ATP. The requirement of acetate thiokinase for ADP may explain the absence of phosphotransacetylase and acetate kinase activities reported by Joyner and Baldwin for *S. ruminantium* HD4 (15). The activity is being studied further to determine if the thiokinase is a single oligomeric protein.

Propionate branch. The enzyme that catalyzes the first step in the production of the propionate, PEP carboxykinase, is greatly affected by changes in growth rate, as the lack of detectable activity at 0.52 h^{-1} illustrates. Synthesis of the enzyme may be regulated at the genetic level, or the enzyme may be covalently modified such that its activity is no longer detectable at this high dilution rate. In either case, carbon flow to propionate must proceed via another enzymatic step. Pyruvate carboxylase (not detected in continuous-culture-grown cells) may perform this function, but if so, the cell loses a net of 1 molecule of ATP per propionate compared with OAA production via PEP carboxykinase. PEP carboxykinase produces 1 molecule of ATP directly in carboxylating PEP to OAA. Pyruvate kinase and pyruvate carboxylase, acting in sequence, produce OAA from PEP with no net ATP production, since the ATP generated in the pyruvate kinase reaction is required for the carboxylation of pyruvate to OAA. The use of an enzyme other than PEP carboxykinase to produce propionate may affect the net production of ATP.

Fumarate is reported to be a terminal electron acceptor in anaerobic electron transport chains in many bacteria (40). Henderson (9) demonstrated that fumarate can reoxidize the cytochrome *b* of *S. ruminantium* that had been previously reduced with H_2. In our laboratory, *S. ruminantium* was observed to grow on H_2 and fumarate as the sole energy source. Large amounts of propionate could be detected in the growth medium (data not shown). This indicated that energy can indeed be derived from fumarate reduction alone (20).

Methylmalonyl-CoA decarboxylase has been shown in *Veillonella alcalescens* and *Propionigenium modestum* to be linked to the production of a Na^+ gradient driven by the exergonic decarboxylation of methylmalonyl-CoA (10, 11). This Na^+ gradient can then be used as an energy source for cellular functions. In *V. alcalescens* the enzyme is associated with the cytoplasmic membrane and is dependent on covalently bound biotin for the decarboxylation reaction (10). The enzyme in *S. ruminantium* has the same properties. We were, however, unable to show a Na^+ dependence for the reaction. This may be so because the enzyme may be similar to that of *V. alcalescens*, which was demonstrated to be highly uncoupled from Na^+ extrusion when methylmalonyl-CoA was in excess (10). In addition, obtaining Na^+-free conditions in our assay was difficult, owing to the ubiquitous nature of this ion.

The [2-^{14}C]lactate labeling studies of Paynter and Elsden (26) led them to conclude that succinate is an intermediate in the production of propionate. Indeed, in our study, succinate was produced in *S. ruminantium* via the action of fumarate reductase (Table 2), as reported earlier (15). The succinate is then extruded from the cell or decarboxylated to propionate via the methylmalonyl-CoA decarboxylase. There are two points of disagreement between our results and those of Joyner and Baldwin (15) concerning the pathway of propionate formation. They did not detect any malate dehydrogenase activity in batch-grown cells, while we detected significant activities of the enzyme in continuous culture (Table 2) and batch-grown cells (results not shown). Joyner and Baldwin reported NADP-dependent malic enzyme activities in batch-grown cells of 0.02 mmol min^{-1} mg of $protein^{-1}$. In our hands, NADP- or NAD-dependent malic enzyme activities were not detected in cell extracts of *S. ruminantium* grown in either continuous or batch culture. The reasons for the discrepancies between the results presented here and those of Joyner and Baldwin are unknown.

Enzyme regulation. Other than PEP carboxykinase, the levels of the other enzymes listed in Table 2 did not change significantly with the dilution rate, suggesting that flow through the pathways may be regulated by changing the catalytic activity of the enzymes. The enzymes that catalyze the first step in a pathway are often sites for regulation. At the PEP branch point, pyruvate kinase, as described in this report, is regulated by FBP and P_i in a manner similar to that found in other organisms (3). Fluctuations in the intracellular concentration of the molecules involved in regulating pyruvate kinase (i.e., P_i and FBP) could play a major role in regulating carbon flux through the pathways shown in Fig. 2.

Bioenergetic considerations. Enzymes have been detected in *S. ruminantium* HD4 that could be involved in three of the four known modes of energy conservation: substrate level phosphorylation (e.g., pyruvate kinase), electron-transport-dependent phosphorylation (fumarate reductase), and decarboxylation linked to Na^+ export (methylmalonyl-CoA decarboxylase) (6, 40). The fourth known mechanism for energy conservation is carrier-mediated proton extrusion, in which intracellular accumulation of a fermentation end product results in the establishment of a concentration gradient. The efflux of the catabolite, in symport with protons, can be used to establish a proton gradient (17). The proton gradient can then be used to synthesize ATP via a H^+-ATPase or to take up nutrients in symport with protons (17). A well-characterized example of such a mode of energy conservation is found in some lactic acid bacteria and in *Escherichia coli*. In these organisms lactate is extruded from the cell in symport with protons (25, 39). *S. ruminantium* HD4 produces large amounts of lactate at fast dilution rates in glucose-limited chemostats and in batch culture with glucose as the carbon source (22). Thus, lactate-dependent carrier-mediated proton extrusion may also provide this organism with an additional source of metabolic energy. Experiments directly linking lactate efflux with proton extrusion must be performed before it can be proved that this is a site of energy conservation in *S. ruminantium*, although the high cell yields found at fast dilution rates, where lactate is the major product, support this hypothesis.

Theoretically, like lactate, succinate might also be extruded from the cell in symport with protons. The results shown in Fig. 1A and B indicate that *S. ruminantium* HD4 obtained its maximum yields coincident with the highest production of succinate at a dilution rate of roughly 0.2 h^{-1}. Russell and Baldwin (29) and Russell (27) also observed a maximum yield at this dilution rate, although they did not detect significant levels of succinate in the chemostat. Although the yield per mole of ATP (Y_{ATP}) is a growth-dependent variable, charges in this parameter can explain the increase and decrease of molar growth yields at dilution rates from 0.1 to 0.3 h^{-1} only to a minor extent, given the extent and direction of the changes observed. The steady-state concentration of each fermentation end product should

reflect the amount of energy harnessed by all energy-conserving sites along the pathway leading to its formation. By this reasoning, increased ATP equivalent formation in the acetate thiokinase and methylmalonyl-CoA decarboxylase reactions could not be responsible for the increase in molar growth yield (Y_M) observed when dilution rates were increased from 0.1 to 0.2 h^{-1}, since the levels of acetate and propionate remained constant and decreased, respectively. The sum of succinate and propionate made, reflecting the amount of ATP equivalents generated via the PEP carboxykinase and fumarate reductase reactions, reached a maximum at a dilution rate of 0.1 h^{-1}; at this dilution rate the Y_M value was at its lowest point. Since an increase in ATP equivalents via known energy-yielding reactions cannot be responsible for the increase in Y_M observed at the dilution rate of 0.2 h^{-1}, a plausible alternative explanation would be that a new site for energy conservation exists somewhere in the pathway of succinate formation, a site totally separate from the reactions leading to propionate formation (e.g., succinate efflux). If this was the case, one would predict that the addition of exogenous succinate to the growth medium should result in decreased molar growth yields. This was indeed observed (T. A. Michel, S. B. Melville, and J. M. Macy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, K100, p. 219).

The high yields that *S. ruminantium* exhibits in continuous culture reported here and by several other investigators (12, 13, 27, 29, 41) have led to much speculation in the literature as to how such yields can be obtained. An attempt was made by Hobson and Wallace (13) to correlate the yields with sites of energy conservation in *S. ruminantium*, but since the pathway of glucose fermentation was known only in part, all sites of energy conservation could not be identified.

High yields in this organism might be due to either a very high Y_{ATP} (gram [dry weight] of cells per mole of ATP produced) or to many sites of energy conservation. The five potential sites of energy-conserving reactions in *S. ruminantium* (substrate level phosphorylation for pyruvate kinase, acetate thiokinase, and PEP carboxykinase; electron transport phosphorylation for fumarate reductase; and Na^+ gradient-linked decarboxylation for methylmalonyl-CoA decarboxylase) suggest that the latter may be the case in this organism.

Previous attempts to determine Y_{ATP} in *S. ruminantium* have been complicated by the fact that values for Y_{ATP} have been shown to vary between microorganisms and to fluctuate in a single organism, depending on such factors as cell composition, growth rate, maintenance energy, and changing fermentation patterns (37, 38). To permit the determination of Y_{ATP} directly from the amounts of fermentation products produced, the exact amount of ATP equivalents produced at each site of energy conservation must be calculated. Such calculations, based on the pathway presented in this report, may allow us to determine a more accurate value of Y_{ATP} for this organism.

Finally, the question arises as to what benefit *S. ruminantium* derives (if any) by shifting from an acetate-propionate fermentation to a lactate fermentation as the growth rate increases. Y_M values at the lowest dilution rates were nearly identical to those at the highest dilution rates (Fig. 1A); thus the energy potential of *S. ruminantium* probably did not increase when it shifted from an acetate-propionate fermentation to a lactate fermentation. Rather, production of lactate by *S. ruminantium* at fast dilution rates may simply allow the cell to produce more ATP per unit of time in order to accommodate the increasing growth rate at the fast dilution rates.

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