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Vitamin B_{12}-Dependent Propionate Production by the Ruminal Bacterium *Prevotella ruminicola* 23†

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When *Prevotella ruminicola* 23 was grown in a defined medium containing a vitamin mixture, significant amounts of propionate were formed. Succinate and acetate were the major fermentation acids produced when vitamins were omitted, and further experiments demonstrated that propionate formation was dependent on vitamin B_{12}. When the organism was grown in continuous culture at dilution rates of less than 0.20 h^{-1}, propionate and acetate were the predominant fermentation products and little succinate was formed when vitamin B_{12} was present. However, at higher dilution rates, propionate formation declined and succinate accumulated. Since cell protein yields were reduced 15 to 25% in the absence of vitamin B_{12}, the pathway for propionate formation may contain an energy-conserving step.

The general catabolic pathways and fermentation products of most predominant ruminal microorganisms have been described (11), but many details regarding specific metabolic steps and the regulation of metabolism remain obscure. Since the ruminal environment is anaerobic, substrate-level phosphorylation reactions are an important means of energy conservation, but many ruminal bacteria also utilize membrane-bound energy conservation mechanisms to retain additional energy during catabolism. However, knowledge concerning energy-yielding reactions in ruminal organisms is incomplete.

Prevotella ruminicola (previously classified as *Bacteroides ruminicola*) is a common gastrointestinal bacterium that utilizes a wide variety of carbohydrates, has high growth yields (13), and tolerates relatively low pH (14). These characteristics provide selective advantages to *P. ruminicola*; it is a successful inhabitant of the competitive gut environment that accounts for up to 19% of all isolates from the rumen (2) and for up to 35% of those from the swine cecum (12). Bryant et al. (2) did not detect propionate as a fermentation product in the original isolation of the organism, but later work by Dehority (5) indicated that *Prevotella ruminicola* subsp. *ruminicola* 23 produced propionate when cultivated in a medium containing 40% rumen fluid. Enzymatic and labelling studies suggested that a nonrandomizing pathway involving acrylate was responsible for propionate formation (19), but these results could not explain decreased succinate production observed in 40% rumen fluid medium (5). Previous work indicated that several *Bacteroides* species required vitamin B_{12} for propionate production (3). Therefore, the effects of vitamin B_{12} on propionate formation and yield were examined.

P. ruminicola 23 was obtained from K. A. Dawson, University of Kentucky. The basal medium contained (per liter) 292 mg of K_2HPO_4; 292 mg of KH_2PO_4; 480 mg of Na_2SO_4; 480 mg of NaCl; 100 mg of $MgSO_4 \cdot 7H_2O$; 64 mg of $CaCl_2 \cdot H_2O$; 530 mg of NH_4Cl; 600 mg of cysteine; 4,000 mg of Na_2CO_3; 1,000 mg of Trypticase Peptone (Becton Dickinson, Cockeysville, Md.); 1 mg of hemin, 1 mM (each) isobutyrate, 2-methylbutyrate, valerate, and isovalerate; microminerals (4); and vitamins (4). Coenzyme B_{12} (5′-deoxyadenosylcobalamine; referred to herein as vitamin B_{12}) was omitted from the vitamin mix in some experiments, and Trypticase Peptone supplied less than 0.45 μg of vitamin B_{12} per liter. Glucose was prepared and autoclaved separately. In some experiments, clarified rumen fluid was added to the basal medium. Rumen contents from a steer which was fed a 60:40 roughage-concentrate diet were squeezed through eight layers of cheesecloth 90 min after feeding and centrifuged three times at low speed (8,000 × *g*, 20 min, 4°C) and then twice at high speed (25,000 × *g*, 15 min, 4°C). Portions were sterilized either by being autoclaved (121°C, 15 min) or by being passed through a nylon membrane filter (pore size, 0.45 μm). The maximum growth rate of batch cultures was determined by monitoring the optical density (600 nm, 18-mm path length) of incubation tubes every 20 min. The medium pH was adjusted to 6.7 with NaOH, the gas phase was 100% CO_2, and incubations were at 39°C.

Continuous culture experiments were conducted by using a New Brunswick F1000 fermentor with a modified 345-ml growth vessel. Steady-state samples were obtained after 4 volumes (>98% turnover) had passed through the growth vessel. Samples were drawn with a syringe, and cells were immediately separated from culture fluid by centrifugation (8,000 × *g*, 20 min, 4°C) and washed once with 0.9% NaCl. Cell-free supernatants and cell suspensions were frozen at −20°C until analysis. Volatile fatty acid concentrations in culture fluid were measured with a Gow Mac gas chromatograph (model 580) equipped with a Supelco 1000 column (1% H_3PO_4, 100/120 mesh). Succinate was measured by gas chromatography after methylation (17). Glucose was measured by an enzymatic method using hexokinase and glucose-6 phosphate dehydrogenase (1). Protein concentrations were determined by the method of Lowry et al. (8) after cells had been hydrolyzed (0.2 N NaOH, 15 min, 100°C).

When supplemental vitamin B_{12} was omitted from the medium, acetate and succinate were the major fermentation products and propionate was not detected (data not shown). Propionate production increased as the concentration of vitamin B_{12} (supplemented as 5′-deoxyadenosylcobalamine) was increased from 0.5 to 50 μg/liter, and there was a stoichiometric decline in succinate levels (Fig. 1). Propionate production did not respond to vitamin B_{12} concentrations greater than 50 μg/liter, and relatively little change in acetate

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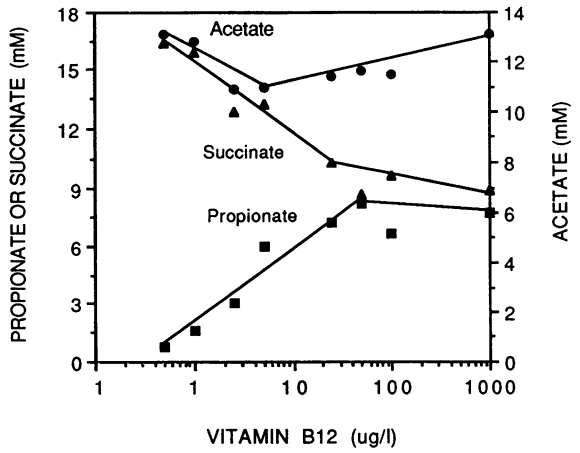

FIG. 1. Effect of vitamin B_{12} supplementation on acid production. Cultures were provided with 11 mM glucose, and cell-free supernatants were collected immediately after growth had ceased.

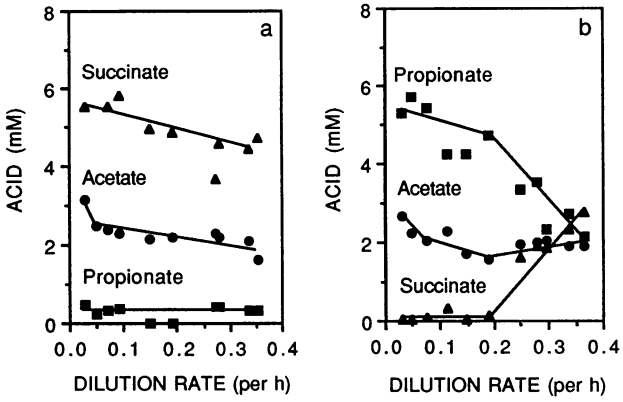

FIG. 3. Acid production by glucose-limited (5.5 mM) continuous cultures in the absence (a) and presence (b) of vitamin B_{12} (50 μg/liter).

formation was observed over the entire range of vitamin concentrations. The maximum growth rate of batch cultures was 0.38 h^{-1} in a medium not supplemented with vitamin B_{12} (Fig. 2), and the growth rate increased to 0.50 h^{-1} as the vitamin concentration was raised to 50 μg/l. Similar results were obtained when the vitamin was added as cyanocobalamin. Since neither cobalt chloride (up to 4.2 μM) nor biotin (up to 500 μg/liter) could satisfy the vitamin B_{12} requirement (data not shown), it appeared that B_{12} might have been the rumen fluid component responsible for propionate formation observed in earlier work (5).

Miller and Wolin (9) stated that B_{12} did not stimulate production of propionate by *P. ruminicola*, but the strain used in their study was not identified. *Prevotella ruminicola* subsp. *brevis* did not produce propionate even in 40% rumen fluid medium (5), and strain differences may account for the discrepancy in propionate production between studies. Several intestinal *Bacteroides* species form propionate at the expense of succinate, and this result was dependent on B_{12} supplementation (3). Vitamin B_{12} was also required for the growth of *Bacteroides fragilis* (18).

Previous experiments (5) indicated that 40% rumen fluid

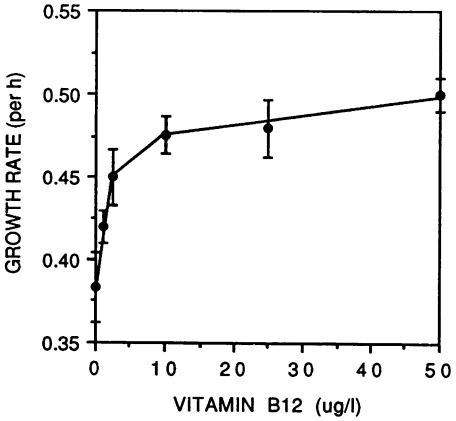

FIG. 2. Effect of vitamin B_{12} supplementation on growth rate. Cultures were provided with 11 mM glucose. Vertical bars represent the standard deviations of three determinations.

was required for propionate formation by *P. ruminicola*. Because the rumen fluid was autoclaved, it was conceivable that heat sterilization affected vitamin composition. Therefore, we incubated the organism in a vitamin B_{12}-deficient medium supplemented with either autoclaved or filter-sterilized rumen fluid. At least 20% rumen fluid was required for detectable propionate formation (data not shown), and the method of sterilization did not affect propionate production. When the basal medium was completely replaced by rumen fluid, propionate production was nearly as great as in media that contained 50 μg of vitamin B_{12} per liter (data not shown). These results suggested that B_{12} concentrations in rumen fluid may be limiting for propionate production by some strains of *P. ruminicola*. Smith and Marston (15) found that more than 95% of ruminal vitamin B_{12} was associated with the bacterial fraction and less than 4% (2.3 μg/liter) was recovered in clarified rumen fluid after centrifugation.

When *P. ruminicola* was grown in continuous culture in the absence of vitamin B_{12}, acetate and succinate were the major fermentation products (Fig. 3a) and virtually no propionate (<5% of total acid production) was detected. Large amounts of propionate and very little succinate were produced at dilution rates of less than 0.20 h^{-1} when the medium was supplemented with vitamin B_{12} (50 μg/liter [Fig. 3b]). However, at higher dilution rates, propionate production declined and succinate accumulated. Bacterial growth yields in continuous culture were consistently higher at all dilution rates when vitamin B_{12} was included in the medium, and omission of the vitamin caused a 15 to 25% decrease in microbial yield (Fig. 4). These differences in yield were most dramatic at lower dilution rates at which propionate production was greatest and succinate had not accumulated.

Stoichiometric declines in succinate associated with increases in propionate production (Fig. 1), in bacterial growth rate (Fig. 2), and in protein yield (Fig. 4), observed in the presence of vitamin B_{12}, suggested that propionate production was associated with the conservation of biologically useful energy. The rumen is often an energy-limited environment, and maximizing energy conservation during fermentation is one strategy ruminal microorganisms use to survive. However, the nature of a putative energy-conserving mechanism in the propionate pathway remains unclear.

Fermentative bacteria use two major pathways in the production of propionate. The direct reductive pathway of propionate formation utilizes several electron transfer reac-

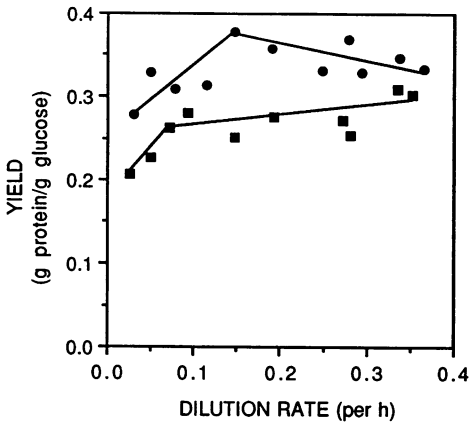

FIG. 4. Bacterial protein yield of glucose-limited (5.5 mM) continuous cultures in the absence (■) and presence (●) of vitamin B_{12} (50 μg/liter).

tions, includes acrylyl-coenzyme A (acrylyl-CoA) as an intermediate, and involves randomization of glucose carbon (6). Joyner and Baldwin (7) found that *P. ruminicola* 23 possessed lactyl-CoA dehydrase, which is a key enzyme in this pathway, and later studies using radioactively labelled glucose indicated that little randomization of label occurred (19). However, since the acrylate pathway involves dehydrogenations rather than carbon transfers, it is hard to imagine that an enzymatic step in this series of reactions requires B_{12}.

Many propionate-producing bacteria form propionate via a randomizing sequence of reactions that includes conversion of succinyl-CoA to methylmalonyl-CoA (6); this carbon transfer rearrangement is catalyzed by a vitamin B_{12}-dependent methylmalonyl mutase. Such a reaction would explain B_{12}-dependent propionate production, the observation that succinate accumulates during B_{12} limitation in batch and continuous cultures (Fig. 1 and 3), and the stoichiometric relationship between succinate and propionate (Fig. 1). White et al. (20) found that *P. ruminicola* possessed *b*-type cytochromes, and fumarate reductase was later detected in cell membrane preparations of the organism (10). These biochemical characteristics are indicative of a randomizing pathway. However, preliminary results from our laboratory suggested that the organism did not possess methylmalonyl-CoA decarboxylase (16), which is another enzyme needed for the production of propionate from succinate. In addition, the presence of acrylate pathway enzymes and the labelling patterns in propionate (19) are difficult to reconcile with the conversion of succinate to propionate. At the present time, the vitamin B_{12} requirement for propionate production remains unexplained.

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