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Francisco Diez-Gonzalez · Daniel R. Bond
Elizabeth Jennings · James B. Russell

Alternative schemes of butyrate production in *Butyrivibrio fibrisolvens* and their relationship to acetate utilization, lactate production, and phylogeny

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Abstract *Butyrivibrio fibrisolvens* strains D1 and A38 produced little lactate, but strain 49 converted as much as 75% of its glucose to lactate. Strain 49 had tenfold more lactate dehydrogenase activity than strains D1 or A38, this activity was stimulated by fructose 1,6-bisphosphate, and had a pH optimum of 6.25. A role for fructose 1,6-bisphosphate or pH regulation of lactate production in strain 49 was, however, contradicted by the observations that very low concentrations (< 0.2 mM) of fructose 1,6-bisphosphate gave maximal activity, and continuous cultures did not produce additional lactate when the pH was decreased. The lactate production of strain 49 was clearly inhibited by the presence of acetate in the growth medium. When strain 49 was supplemented with as little as 5 mM acetate, lactate production decreased dramatically, and most of the glucose was converted to butyrate. Strain 49 did not possess butyrate kinase activity, but it had a butyryl-CoA/acetate CoA transferase that converted butyryl-CoA directly to butyrate, using acetate as an acceptor. The transferase had a low affinity for acetate (K_m of 5 mM), and this characteristic explained the acetate stimulation of growth and butyrate formation. Strains D1 and A38 had butyrate kinase but not butyryl-CoA/acetate CoA transferase, and it appeared that this difference could explain the lack of acetate stimulation and lactate production. Based on these results, it is unlikely that *B. fibrisolvens* would ever contribute significantly to the pool of ruminal lactate. Since relatives of strain 49 (strains Nor37, PI-7, VV1, and OB156, based on 16S rRNA sequence analysis) all had the same method of butyrate production, it appeared that butyryl-CoA/acetate CoA transferase might be a phylogenetic characteristic. We obtained a culture of strain B835 (NCDO 2398) that produced large amounts of lactate and had butyryl-CoA/acetate CoA transferase activity, but this strain had previously been grouped with strains A38 and D1 based on 16S rRNA sequence analysis. Our strain B835 had a 16S rRNA sequence unique from the one currently deposited in GenBank, and had high sequence similarity with strains 49 and Nor37 rather than with strains A38 or D1.

F. Diez-Gonzalez · D. R. Bond · E. Jennings · J. B. Russell (✉)
Wing Hall, Cornell University, Ithaca, NY 14853, USA
e-mail: jbr8@cornell.edu
Tel. +1-607-255-4508; Fax +1-607-255-3904

J. B.Russell
Agricultural Research Service, USDA, Ithaca, NY 14853, USA

Introduction

The rumen is well-buffered with bicarbonate, but the rate of fermentation can at times be so rapid that ruminal pH declines (Slyter 1976). Ruminal acidosis causes decreased food intake and even death of the animal. Because lactate is a tenfold stronger acid than the volatile fatty acids, the growth of lactate-producing bacteria promotes ruminal acidosis (Hungate et al. 1952). While some strains of *Butyrivibrio fibrisolvens* can produce large amounts of lactate (Bryant 1984), and acetate, which is normally present in the rumen at high (> 50 mM) concentrations, can increase both the growth rate (Roché et al. 1973) and the butyrate production rate (Latham and Legakis 1976) of lactate-producing butyrivibrios. Such interactions have confounded estimates of the contribution by *B. fibrisolvens* to ruminal lactate production.

The metabolism of *B. fibrisolvens* has not been extensively studied, but lactate-producing strains have lactate dehydrogenases that are stimulated by the glycolytic intermediate fructose 1,6 bisphosphate (Van Gylswyck 1977). Miller and Jenesel (1979) have demonstrated that *B. fibrisolvens* D1 has butyrate kinase activity, but this strain does not produce large amounts of lactate and its growth is not stimulated by acetate (Bryant and Robinson 1962). Acetate-stimulated strains incorporate label from ^{14}C-acetate into butyrate, but the pathway of this incorporation has not been defined (Van Gylswyk 1976).

Clostridia have two distinctly different pathways of butyrate production (Gottschalk 1986). Saccharolytic strains usually utilize a scheme employing butyrate kinase, but *Clostridium kluyverii* lacks butyrate kinase activity when

it is growing on ethanol, and utilizes a butyryl-CoA/acetate CoA transferase that produces butyrate directly from butyryl-CoA. CoA is transferred to acetate, making butyrate production dependent on the presence of acetate in the medium (Gottschalk 1986).

Strains of *B. fibrisolvens* form a heterogeneous group of bacteria that ferment a wide array of substrates. Hungate (1966) proposed that non-lactate-producing *B. fibrisolvens* strains should be reclassified as *B. alactacidigens*, but subsequent taxonomic descriptions have deleted this designation (Bryant 1984). Recent work based on 16S rRNA gene sequence analysis indicated that ruminal *B. fibrisolvens* strains clearly fell into two phylogenetic groups (Forester et al. 1996; Willems et al. 1996), but the authors have concluded that "phenotypic data that support division of the genus *Butyrivibrio* are not available" (Willems et al. 1996). Lactate-producing, acetate-stimulated strains are generally distinct from non-lactate-producing, non-acetate stimulated strains, but some exceptions have been noted.

Based on the observation that lactate-producing strains of *B. fibrisolvens* are generally acetate-stimulated, we hypothesized that they might produce butyrate via butyryl-CoA/acetate CoA transferase rather than butyrate kinase. Since lactate utilization and acetate stimulation have sometimes been correlated with current understanding of phylogenetic differences (Shane et al. 1969), we also hypothesized that butyryl-CoA/acetate CoA transferase might be related to the phylogenetic placement of these organisms.

Materials and methods

Cells and growth

B. fibrisolvens strains D1, A38, 49, Nor37, PI-7, VV1, OB156, and B835 were obtained from M.A. Cotta, ARS/USDA (Peoria. Ill., USA). Cultures were grown anaerobically at 39 °C in basal medium containing (per liter) 292 mg K_2HPO_4, 292 mg KH_2PO_4, 480 mg $(NH_4)_2SO_4$, 480 mg NaCl, 100 mg $MgSO_47H_2O$, 64 mg $CaCl_22H_2O$, 600 mg cysteine hydrochloride, 10 g trypticase (BBL Microbiology, Cockeysville, Md., USA), 2.5 g yeast extract, branched-chain volatile fatty acids (1 mmol each of isobutyrate, isovalerate, and 2-methylbutyrate), plus hemin, vitamins, and microminerals (Cotta and Russell 1982). Glucose and sodium acetate were prepared as separate solutions and were added separately after autoclaving. The growth rate was estimated from the increase in optical density (600 nm, 1-cm cuvettes). *B. fibrisolvens* strain 49 was also grown in glucose-limited continuous culture (5.55 mM glucose and 40 mM sodium acetate added to the medium reservoir) under O_2-free CO_2 at a dilution rate of 0.1 h^{-1} (190-ml culture vessel, 39 °C). The pH of the medium reservoir was adjusted by addition of HCl, and at least a 98% turnover of the medium through the continuous-culture vessel occurred between samplings (approximately four culture vessel volumes). All cultures were harvested by centrifugation (10,000 × *g*, 5 °C, 10 min), and the cell-free supernatants were stored at –15 °C. The cell pellets were washed twice with 0.9% NaCl and stored at –15 °C.

Cell extracts and enzyme assays

Batch cultures (500 ml) were harvested anoxically by centrifugation (10,000 × *g*, 5 °C, 10 min) and were transferred to an anoxic glove box (95% CO_2 and 5% H_2; Coy Laboratory Products). Cells were washed twice in 50 mM Tris-HCl (5,000 × *g*, 4 °C, 10 min, pH 7.0) and disrupted by sonication (Branson model 200, microtip; maximum output, 7; 50% duty cycle; 10 min, 0 °C). The cell extract was placed in a sealed polycarbonate centrifuge tube (40 ml), and cell debris was removed by centrifugation (10,000 × *g*, 4 °C, 10 min). The cell-free extract was transferred anoxically to a sealed vial and stored at –15 °C.

Lactate dehydrogenase (EC 1.1.1.27) activity was determined by measuring the disappearance of NADH at 340 nm (ε of 6.22 mM^{-1} cm^{-1}) (Freier and Gottschalk 1987). Fructose 1,6-bisphosphate (1 mM) was added to each assay unless otherwise stated. Butyryl-CoA/acetate CoA transferase (EC 2.8.3.8) activity was measured by following the decrease in butyryl-CoA concentration at 233 nm according to Barker et al. (1955). Acetate kinase (EC 2.7.2.1) and butyrate kinase (EC 2.7.2.7) were assayed by hydroxamate formation (Rose 1955).

Determination of 16S rRNA sequence

Chromosomal DNA was purified from strain B835 using a FastDNA Spin Kit (Bio101, Vista, Calif., USA), and the gene for the 16S rRNA was amplified using conserved primers. The amplified fragment corresponded to positions 27–1492 of the *Escherichia coli* 16S rRNA gene (Lane 1991). PCR products were cloned immediately after PCR using a TA Cloning Kit (Invitrogen, Carlsbad, Calif., USA), and clones were submitted to the Cornell Biotechnology Center for sequencing using two primers based on the plasmid cloning site and four primers based on internal conserved regions (Lane 1991). Sequences were assembled and edited using the SeqMan component of the Lasergene package and were submitted to GenBank (accession no. AF125217). The 16S rRNA sequence from strain B835 was aligned with other known *Butyrivibrio* 16S rRNA sequences using the Clustal method (MegAlign, Lasergene, DNASTAR, Madison, Wis., USA), and similarity with selected *Butyrivibrio* sequences was calculated using the Similarity Matrix (version 1.1) service of the Ribosomal Database Project (Maidak et al. 1997).

Other analyses

Bacterial protein in cell suspensions was measured by the method of Lowry et al. (1951) after the cells had been heated to 100 °C in 0.2 N NaOH for 15 min. Bovine serum albumin was the standard. Glucose, formate, lactate, acetate, and butyrate in cell-free supernatants were analyzed by high-pressure liquid chromatography with a Beckman 334 liquid chromatograph equipped with a model 156 refractive index detector and a BioRad HPX-87H organic acid column. The sample size was 20 µl, the elutant was 0.0065 M H_2SO_4, the flow rate was 0.5 ml/min, and the column temperature was 50 °C. Gas samples for hydrogen measurements were taken from the head space of sealed incubation tubes and were analyzed using a Gow Mac 550 thermoconductivity gas chromatograph (Carbosieve S8100 mesh column; Supelco, Bellefonte, Pa., USA).

Results

Fermentation end-products and growth rate

B. fibrisolvens strains A38 and D1 never produced significant amounts of lactate, not even if acetate was omitted from the growth medium (Fig. 1). Strain 49 produced large amounts of lactate from glucose, but only if acetate was omitted from the basal medium. Strain 49 utilized a small amount of acetate when acetate was provided, but strains A38 and D1 always produced acetate. Acetate addition increased the growth rate of strain 49 by more than

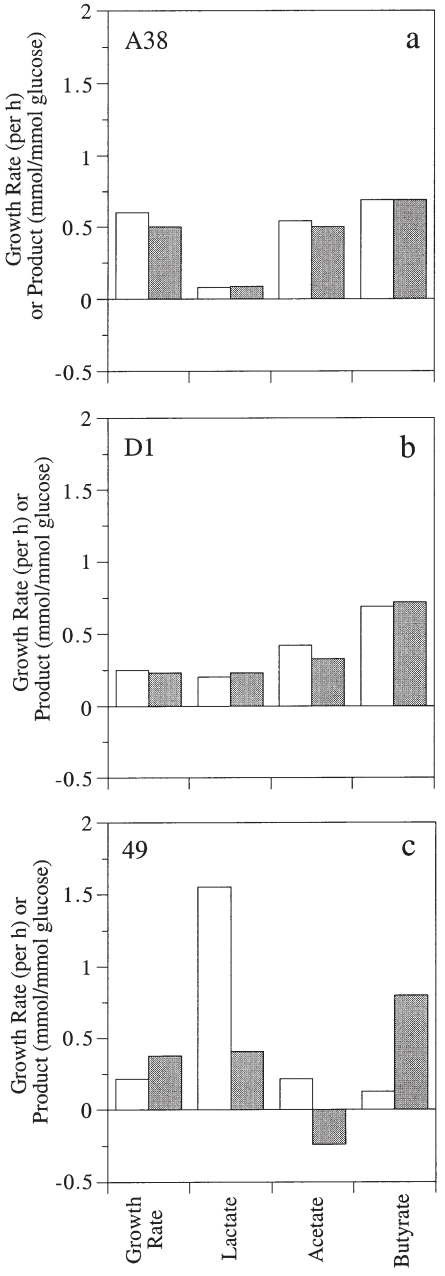

Fig. 1 The specific growth rate and fermentation endproducts of **a** *Butyrivibrio fibrisolvens* A38, **b** D1, and **c** 49 in basal medium *(open bars)* or basal medium that was supplemented with 40 mM sodium acetate *(shaded bars)*. Endproducts are expressed as mmol product per mmol glucose fermented

50%, but acetate had much less impact on the growth rate of strains A38 and D1. The amount of acetate needed to decrease the lactate production of strain 49 was less than 5 mM (Fig. 2 a). When lactate production decreased, butyrate, formate, and hydrogen production increased (Fig. 2 a, b). Strains Nor37, PI-7, VV1, OB156, and B835 were all similar to strain 49 in phenotype, and the presence of acetate caused a shift from lactate to butyrate production (data not shown).

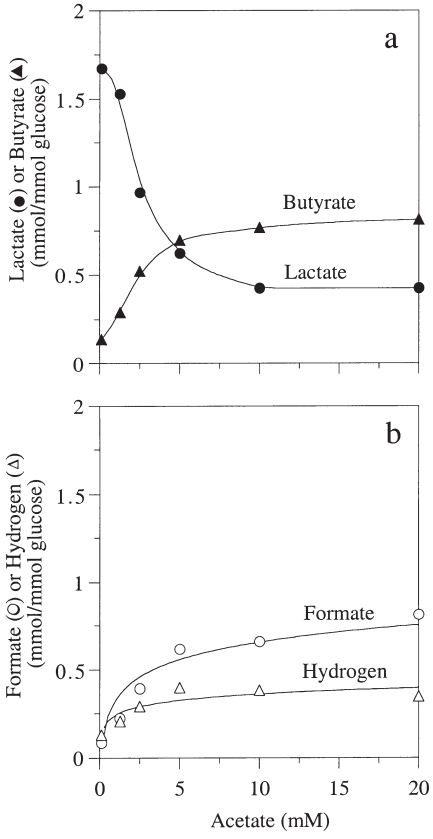

Fig. 2 The effect of acetate concentration on **a** the lactate (●) and butyrate (▲) production or **b** the formate (○) and hydrogen (△) production of *Butyrivibrio fibrisolvens* strain 49

Enzymatic studies

Strain 49 had at least tenfold more lactate dehydrogenase activity than did strains D1 and A38 (Fig. 3 a), and the pH optimum was approximately 6.25. The lactate dehydrogenase of strain 49 was stimulated by fructose 1,6-bisphosphate, but the concentration needed for maximal velocity was only 0.2 mM (Fig. 3 b). The lactate dehydrogenase activities of strains D1 and A38 appeared lower when fructose 1,6-bisphosphate was not present, but the differences were not significant. Strains D1 and A38 possessed butyrate kinase and acetate kinase activities, but butyrate kinase was not detected in strain 49 (Table 1). Strains D1 and A38 had no detectable butyryl-CoA/acetate CoA transferase activities, but strain 49 had high butyryl-CoA/acetate CoA transferase activity, even when grown in the absence of acetate. The butyryl-CoA/acetate CoA transferase of strain 49 required relatively large amounts of acetate, and the K_m was 5 mM (Fig. 4). Strains Nor37, PI-7, VV1, OB156, and B835 all lacked butyrate kinase and had significant butyryl-CoA/acetate CoA transferase activities (Table 1).

Continuous cultures

When strain 49 was grown at pH 6.8 in glucose-limited continuous culture at a dilution rate of 0.1 h^{-1} with 40 mM

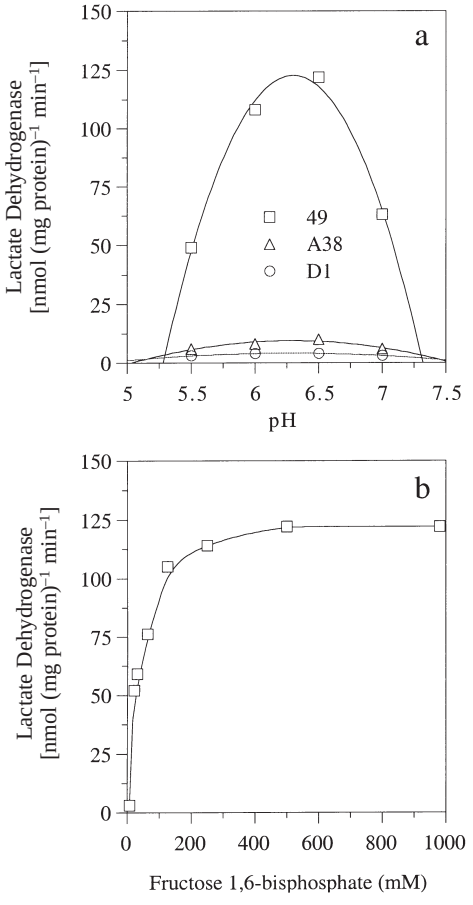

Fig. 3 The effect of pH on the lactate dehydrogenase of *Butyrivibrio fibrisolvens* D1 (○), A38 (△), and 49 (□). **b** The effect of fructose 1,6-bisphosphate concentration on the lactate dehydrogenase of strain 49

Table 1 Enzymatic activities of *Butyrivibrio fibrisolvens* strains. Cultures were grown on 20 mM glucose with 40 mM acetate added to the medium. Enzyme assays were performed at pH 7.0, and activities are μmol (mg protein)$^{-1}$ min^{-1}. The results are the mean of at least two measurements and the standard deviation was less than 10% in all cases

Strain	Acetate kinase	Butyrate kinase	Butyryl-CoA/ acetate-CoA transferase
D1	0.04	0.22	< 0.01
A38	0.14	0.88	< 0.01
49	0.39	< 0.01	1.90
B835[a]	0.40	< 0.01	0.99
Nor37	0.68	< 0.01	0.86
PI-7	2.13	< 0.01	0.23
VV1	1.14	< 0.01	0.73
OB156	0.88	< 0.01	0.82

[a] This culture of strain B835 is unique from strain B835 (NCDO 2398) sequenced by Willems et al. (1996) (see Results)

sodium acetate, the products of fermentation were similar to those of batch cultures grown in the presence of acetate (Fig. 5). When the pH was decreased with HCl, butyrate

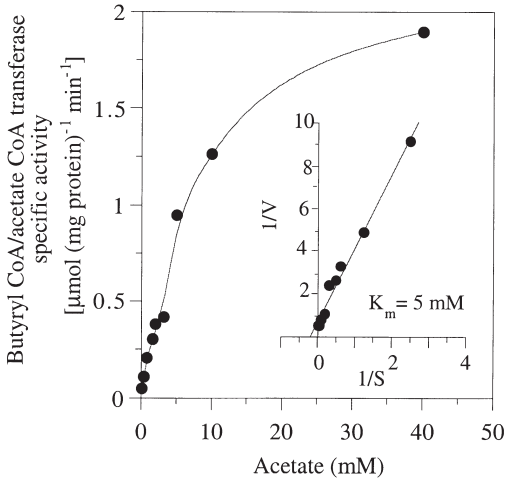

Fig. 4 The effect of acetate concentration on the butyryl-CoA/acetate CoA transferase of *Butyrivibrio fibrisolvens* 49. A Lineweaver-Burk plot is shown in the *inset*

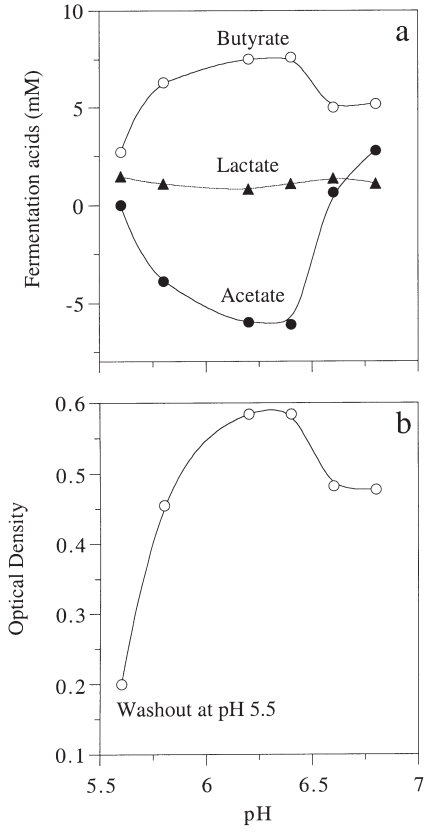

Fig. 5 The effect of **a** pH on butyrate (○), lactate (▲), and acetate (●) or **b** optical density (○) for *Butyrivibrio fibrisolvens* strain 49 grown under glucose-limited conditions with 40 mM sodium acetate

production increased slightly at the expense of acetate, but lactate production was unaffected. At pH 5.6, the lowest pH tolerated by strain 49, butyrate production declined, but lactate did not increase.

16 rRNA gene sequencing

The gene from chromosomal DNA coding for the 16S rRNA of our *B. fibrisolvens* strain B835 had a sequence that was significantly different (similarity only 87%) from the one originally reported for strain B835 by Willems et al. (1996). Similarity between our B835 strain and strains A38 and D1 was also relatively low (< 88%). When the sequence was aligned and compared to all available *B. fibrisolvens* 16S rRNA genes, the greatest similarity was found to *B. fibrisolvens* strains Nor37 and 49 (99.7% and 96.6%, respectively).

Discussion

In many low G+C, gram-positive anaerobes, lactate dehydrogenase is affected by intracellular fructose 1,6-bisphosphate and pH (Garvie 1980). For instance, when the glycolytic rate of *Streptococcus bovis* decreases, intracellular fructose 1,6-bisphosphate decreases, the lactate dehydrogenase becomes less active, and lactate production is reduced nearly tenfold (Bond and Russell 1996). Glycolytic rate-dependent regulation of lactate production can be counteracted by decreases in intracellular pH (Russell and Hino 1985). When the intracellular pH is low, lactate dehydrogenase is active even if fructose 1,6-bisphosphate is unavailable, making lactate the dominant endproduct under acidic conditions at slow growth rates.

B. fibrisolvens strains D1 and A38 produced little lactate and had lactate dehydrogenase activities that were less than 10 nmol (mg protein)$^{-1}$ min^{-1}. Strain 49 had lactate dehydrogenase activity greater than 100 nmol (mg protein)$^{-1}$ min^{-1}, but lactate was only a significant endproduct when acetate concentrations were lower than 5 mM. The lactate dehydrogenase of strain 49 was fructose-1,6-bisphosphate-activated, but the concentration of fructose 1,6-bisphosphate required for maximal activity was less than 1 mM. The lactate dehydrogenase activity was also stimulated when the assay pH was decreased from 7 to 6, but continuous cultures that were provided with acetate produced little lactate even at low pH values. Based on these results and on the fact that ruminal acetate concentrations are always greater than 5 mM, it is unlikely that either type of *B. fibrisolvens* strain would ever contribute significantly to the pool of ruminal lactate.

Many low G+C, gram-positive, lactate-producing bacteria can produce acetate, but acetate is not normally considered to be a regulator of lactate production. The lactate production of *B. fibrisolvens* strain 49 (and of strains Nor37, VV1, P1–7, OB156, and B835) was clearly inhibited by acetate. When acetate was present and the pH was greater than 6.5, butyrate was the dominant endproduct, acetate utilization was minimal, and the cultures produced large amounts of formate and hydrogen. Latham and Legakis (1976) had indicated that conversion of acetate to butyrate was a mechanism for disposal of reducing equivalents. We never observed significant acetate utilization in batch culture, and continuous cultures only consumed acetate when the pH was acidic. Based on these results, acetate is absolutely required as a cofactor for butyrate production in these strains. Under some conditions, presumably when hydrogen or formate production is inhibited, this pathway can also be used to produce butyrate from acetate as a mechanism for disposal of reducing equivalents.

It has generally been assumed that butyrate kinase is the dominant pathway of butyrate production in clostridia (MacFarlane and Gibson 1996). Miller and Jenesel (1979) have indicated that *B. fibrisolvens* D1 has high butyrate kinase activity, and we obtained similar results with strains D1 and A38. Strain 49 produced as much butyrate as strains D1 and A38 if acetate was available, but butyrate kinase was not detected. Instead, strain 49 had a butyryl-CoA/acetate-CoA transferase reaction. This pathway does not negatively affect the organism's ATP yield

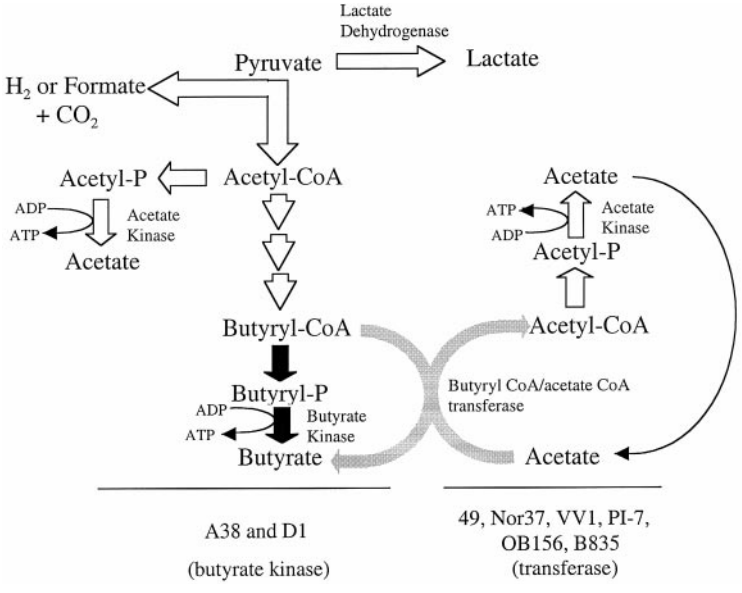

Fig. 6 Proposed pathways of butyrate production for strains A38 and D1 *(dark arrows)* vs strains 49, Nor37, VV1, PI-7, OB156, and B835 *(shaded arrows)*. *Open arrows* represent enzymes common to all strains

since when butyryl-CoA is converted to acetyl-CoA, butyrate can be produced directly, while ATP is subsequently generated via acetate kinase (Fig. 6).

The observation that strain 49 had butyryl-CoA/acetate CoA transferase but not butyrate kinase activity is consistent with this strain's acetate stimulation and may also explain acetate stimulation in other ruminal organisms. Schulman and Valentino (1976) have reported that a purified CoA transferase from *Megasphaera elsdenii* has a relatively low affinity for acetate, and Hino et al. (1991) have observed that butyrate production does not occur in *M. elsdenii* until acetate is present in the medium. The butyryl-CoA/acetate CoA transferase of *B. fibrisolvens* strain 49 had a K_m for acetate of 5 mM, and the shift from lactate to butyrate production required less than 5 mM extracellular acetate (Fig. 2).

It has long been recognized that label from ^{14}C-acetate rapidly enters the ruminal butyrate pool, but Leng (1970) has noted that acetate activation to acetyl-CoA, and net acetate consumption, would be "a net loss of energy to an organism synthesizing butyrate from acetate", especially since acetate activation in most organisms requires hydrolysis of ATP to AMP. However, if butyrate is produced via butyryl-CoA/acetate CoA transferase and acetyl-CoA is generated from acetate, label from ^{14}C-acetate can be exchanged into butyrate (Fig. 6). If no net acetate utilization occurs (acetyl-CoA is excreted via acetate kinase), the energy for acetate "activation" is recovered and does not represent a metabolic drain.

Comparison of previous 16S rRNA analyses (Willems et al. 1996; Forester et al. 1997) and our enzymatic data indicated that *B. fibrisolvens* strains having butyryl-CoA/acetate CoA transferase activity formed a phylogenetic cluster, but our strain B835 was an exception. However, the DNA-DNA hybridizations of Mannarelli (1988), the cell wall structure analysis of Dibbayawan et al. (1985), and our 16S rRNA sequencing analyses indicated that strain B835 is similar to strain Nor37, a strain that is only distantly related to strains A38 and D1. Based on these comparisons, there are probably two "B835" strains in current use: one that is similar to strains A38 and D1, and one that clusters with strain 49.

Willems et al. (1996) have concluded that "phenotypic data that support division of the genus *Butyrivibrio* along phylogenetic lines are not available", but this conclusion is at least in part based on strain B835. Shane et al. (1969) have proposed that *B. fibrisolvens* strains could be partitioned into those that "produced appreciable amounts of lactate" and those that "produced acetate but little or no lactate", but some strains were ambiguous in this phenotype. Because *B. fibrisolvens* strains appear to utilize either butyryl-CoA/acetate CoA transferase or butyrate kinase, but not both, it is conceivable that these pathways of butyrate production provide a connection between the phylogeny and physiology of these bacteria.

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