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Pathway of Succinate and Propionate Formation in *Bacteroides fragilis*

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Cell suspensions of *Bacteroides fragilis* were allowed to ferment glucose and lactate labeled with ^{14}C in different positions. The fermentation products, propionate and acetate, were isolated, and the distribution of radioactivity was determined. An analysis of key enzymes of possible pathways was also made. The results of the labeling experiments showed that: (i) *B. fragilis* ferments glucose via the Embden-Meyerhof pathway; and (ii) there was a randomization of carbons 1, 2, and 6 of glucose during conversion to propionate, which is in accordance with propionate formation via fumarate and succinate. The enzymes 6-phosphofructokinase (pyrophosphate-dependent), fructose-1,6-diphosphate aldolase, phosphoenolpyruvate carboxykinase, malate dehydrogenase, fumarate reductase, and methylmalonyl-coenzyme A mutase could be demonstrated in cell extracts. Their presence supported the labeling results and suggested that propionate is formed from succinate via succinyl-, methylmalonyl-, and propionyl-coenzyme A. From the results it also is clear that CO_2 is necessary for growth because it is needed for the formation of C4 acids. There was also a randomization of carbons 1, 2, and 6 of glucose during conversion to acetate, which indicated that pyruvate kinase played a minor role in pyruvate formation from phosphoenolpyruvate. Phosphoenolpyruvate carboxykinase, oxaloacetate decarboxylase, and malic enzyme (nicotinamide adenine dinucleotide phosphate-dependent) were present in cell extracts of *B. fragilis*, and the results of the labeling experiments agreed with pyruvate synthesis via oxaloacetate and malate if these acids are in equilibrium with fumarate. The conversion of [2-^{14}C]- and [3-^{14}C]lactate to acetate was not associated with a randomization of radioactivity.

Bacteroides fragilis is an important anaerobe of the human intestine. It has a CO_2-dependent metabolism similar to that of its rumen counterpart, *B. ruminicola* (15). Previous work with *B. fragilis* has shown that, when grown on glucose in a minimal medium under an atmosphere of 100% CO_2, the growth rate is slow (g = 8 h) and the yield of cells is low (Y_m = 18) (15). The major fermentation products formed under these conditions are fumarate and lactate, with approximately 1 mol of fumarate formed per mol of glucose fermented (15). When hemin (2 μg/ml) is included in the same medium, growth is faster (g = 2 h), the yield of cells is greater (Y_m = 47), and the major fermentation products are propionate, succinate, and acetate (15). It was also found that the presence of hemin in the growth medium is necessary for the formation of reduced nicotinamide adenine dinucleotide (NADH):fumarate oxidoreductase (EC 1.3.1.6) and a membrane-bound cytochrome *b*. It was suggested that hemin is needed for the synthesis of the cytochrome, which is part of a primitive electron transport system that functions in the generation of energy during the flow of electrons from NADH to fumarate. Thus, in the absence of hemin *B. fragilis* is forced to subsist on ATP generated solely via substrate-level phosphorylation, whereas in the presence of hemin additional energy is formed by electron transport phosphorylation.

Fumarate reductase activity is not detected in *B. fragilis* when grown in the absence of hemin, which explains why formation of succinate is hemin dependent. The same finding also suggests that succinate may be a precursor of propionate, as the latter is found only in "hemin" cultures. If this is the case, then propionate formation in *B. fragilis* might occur via a pathway with succinyl-coenzyme A (CoA), methylmalonyl-CoA, and propionyl-CoA as intermediates; this pathway could be similar either to that in *Propionibacterium shermanii* (35) or to that in *Veillonella* (18). In *P. shermanii* the conver-

sion of methylmalonyl-CoA to propionyl-CoA is catalyzed by transcarboxylase (EC 2.1.3.1), whereas in *Veillonella* this conversion is carried out by methylmalonyl-CoA decarboxylase (EC 4.1.1.41).

Another possible pathway for the formation of propionate by *B. fragilis* would be the reduction of lactate via lactyl-CoA, acrylyl-CoA, and propionyl-CoA. This pathway has been demonstrated in *Megasphera elsdenii* (5, 6, 29) and *B. ruminicola* (32). However, this pathway is independent of fumarate reductase, and there is not a known requirement for a cytochrome. Thus, if propionate is formed via this pathway, the requirement that hemin must be present for the formation of propionate is not easily understood.

Unlike the pathways of propionate formation found in *P. shermanii* and *Veillonella*, where fumarate and succinate are symmetrical intermediates, none of the intermediates of the "acrylate" pathway is symmetrical. This difference in pathways is reflected in the products formed when glucose, labeled in different carbons, is fermented. If fumarate and succinate are intermediates in propionate formation, carbons 1, 2 and 5, 6 of glucose will randomize so that both the second and third carbons of propionate will be labeled. However, no randomization of these carbons would occur if propionate were formed from lactate via the acrylate pathway.

In this paper we report results of fermentation by *B. fragilis* of glucose variously labeled with ^{14}C and of [2-^{14}C]- and [3-^{14}C]lactate, as well as analyses of some key enzymes of possible pathways. The labeling patterns in the products, acetate and propionate, plus the enzyme analyses have allowed us to conclude that *B. fragilis* ferments glucose via the Embden-Meyerhof pathway, that CO_2 is needed for the formation of C4 acids from phosphoenolpyruvate (PEP), that propionate is formed from succinate, and that pyruvate is formed not only directly from PEP but also from C4 acids.

MATERIALS AND METHODS

Growth of bacteria. *B. fragilis* ATCC 23745 was grown anaerobically (100% CO_2) in glucose minimal medium containing 2 μg of hemin per ml as described previously (15).

Fermentation of radioactive substrates by cell suspensions and analytical methods. Cells in the mid-log phase of growth were harvested by centrifugation for 20 min at 10,000 × *g* (4°C). To prevent breakage, the cells were not washed. A 10-g (wet weight) amount of cells was suspended in 15 ml of anaerobic medium lacking hemin, vitamin B_{12}, and glucose. By means of a syringe, 1.5-ml (1-g) aliquots of this suspension were injected into serum-stoppered 50-ml Erlenmeyer flasks, each containing 20 ml of anaerobic medium (lacking vitamin B_{12} and hemin), an atmosphere of 100% CO_2, and either 1 mmol of glucose, 0.4 mmol of lactate, or a combination thereof. After 4.5 h of incubation at 37°C, the fermentations were stopped by the addition of acid (1 ml of 10 N H_2SO_4) to each flask. CO_2 within each flask was collected in a pre-weighed trap, containing 3 ml of bicarbonate-free 4 N sodium hydroxide, by bubbling N_2 through the acidified fermentation mixture and the trap. After the collection the trap was again weighed, and the weight difference was used to estimate the amount of CO_2 that was present in the fermentation vessel. Glucose utilization was estimated by the blood sugar GOD-Perid method of Boehringer, Mannheim GmbH. Lactate was analyzed by the method of Hohorst and Bergmeyer (10). Acetic and propionic acids were obtained by ether extraction of the acidified fermentation mixture followed by chromatographic purification on Celite (28). Before degradation by the Schmidt procedure (20), these acids were diluted with carrier acetate and propionate and further purified by steam distillation. Total oxidation of radioactive samples was done according to Van Slyke and Folch (30). Counting of CO_2 was done as $BaCO_3$ suspended in Scinti Verse Universal LSC cocktail (Fisher), in a Beckman LS-100C liquid scintillation system. Liquid scintillation counting was also done using Unisolv (Koch-Light Lab) and a Packard model 3375 counter.

Preparation of cells. Cells harvested as described earlier were washed twice with cold minimal salts solution having the same composition as the medium but lacking $NaHCO_3$, cysteine·HCl, vitamin B_{12}, hemin, and glucose. The washed cells were suspended in one of the following buffers (2 g/10 ml of buffer): 50 mM potassium phosphate (pH 7.0) for assay of malate dehydrogenase (EC 1.1.1.37), malic enzyme (nicotinamide adenine dinucleotide [NAD]) (EC 1.1.1.39), malic enzyme (NAD phosphate [NADP]) (EC 1.1.1.40), and oxaloacetate decarboxylase (EC 4.1.1.3); 50 mM potassium phosphate (pH 7.0) plus 5 mM $MgCl_2$ plus 0.1 M sucrose for assay of membrane-bound lactate dehydrogenase (2,6-dichlorophenolindophenol); 100 mM potassium phosphate (pH 6.8) for assay of transcarboxylase (EC 2.1.3.1); 100 mM potassium phosphate (pH 7.0) for assay of PEP carboxyphosphotransferase (EC 4.1.1.38), PEP carboxykinase (EC 4.1.1.49), malate-lactate transhydrogenase (EC 1.1.99.7), lactate dehydrogenase (NAD) (EC 1.1.1.27), and methylmalonyl-CoA mutase (EC 5.4.99.2); 20 mM imidazole-hydrochloride (pH 7.0) for assay of pyruvate kinase (EC 2.7.1.40) and pyruvate orthophosphate dikinase (EC 2.7.9.1); 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) for assay of pyruvate carboxylase (EC 6.4.1.1) and PEP carboxykinase; or 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6) for assay of 6-phosphofructokinase (EC 2.7.1.11), pyrophosphate-dependent 6-phosphofructokinase, and fructose-1,6-diphosphate aldolase (EC 4.1.2.13). The cell suspension was then passed at 0°C through a French press (pressure, 66 to 80 kg/cm^3). Unbroken cells were removed by centrifugation for 10 min at 15,000 × *g*. The extract was then centrifuged at 80,000 × *g* for 2 h to separate the membranes from the soluble enzymes. The membranes were washed once before being suspended in the appropriate buffer. Be-

fore soluble enzymes were assayed, the soluble enzyme fraction was passed through a Sephadex G-25 column (1.2 by 13.5 cm) previously equilibrated with the buffer used to prepare broken cells. Protein was determined by the method of Lowry et al. (14).

Enzyme assays. Oxaloacetate decarboxylase was assayed manometrically according to the method of Herbert (9). Methylmalonyl-CoA mutase was assayed by using the method of Kellermeyer and Wood (11), except that 1 mM succinyl-CoA replaced the succinyl-CoA generating system. Also, methylmalonyl-CoA racemase was not included, since it was not available to us. This meant that quantitative estimates of enzyme activity were not possible because the transcarboxylase uses only the S form of methylmalonyl-CoA; thus it was only possible to ascertain whether the enzyme was present or not.

The assay for PP_i-dependent 6-phosphofructokinase was that of Reeves et al. (21). The NAD-dependent malic enzyme was assayed according to the method of Sanwal (22), and the NADP-dependent malic enzyme was assayed using Sanwal and Smando's method (23), except that the 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5) in the reaction mixture was replaced by 50 mM HEPES (*N*-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid) (pH 7.0). The method used to assay pyruvate kinase was that of Kornberg and Malcovati (12), and pyruvate orthophosphate dikinase was assayed according to the method of South and Reeves (27). The transcarboxylase assay was carried out in the manner described by Wood et al. (37), and a method of Wood et al. was also used to assay PEP carboxytransphosphorylase (36). The assay method used to estimate pyruvate carboxylase was that described by von Hugo et al. (31). Malate-lactate transhydrogenase activity was determined according to the method of Allen (2). PEP carboxykinase was assayed by using the method of Scardovi and Chiappini (25).

The oxidation of lactate, catalyzed by NAD-dependent lactate dehydrogenase, was measured by the method of Wittenburger and Fulco (34), which uses acetylpyridine adenine dinucleotide in place of NAD. The reverse reaction, reduction of pyruvate, contained the following in a volume of 1 ml: potassium phosphate buffer (pH 7.5), 13.2 mM; pyruvate, 6.6 mM; and NADH, 0.12 mM. The oxidation of NADH was followed at 340 nm. The NAD-independent lactate dehydrogenase was assayed by using the following reaction mixture (1-ml volume): potassium phosphate (pH 7.6), 50 mM; 2,6-dichlorophenolindophenol, 0.15 mM; and D-(−)-lactate or L-(+)-lactate, 1 mM.

The assay mixture for 6-phosphofructokinase (3) contained the following in a final volume of 1 ml: tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6), 50 mM; NADH, 0.12 mM; fructose-6-phosphate, 1 mM; ATP, 1 mM; $MgCl_2$, 10 mM; NH_4Cl, 2 mM; and excess fructose-1,6-diphosphate aldolase, triosephosphate isomerase, and α-glycerolphosphate dehydrogenase. Aldolase was assayed in a mixture of the following composition (1 ml final volume) (4): tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.6), 50 mM; NADH, 0.12 mM; fructose-1,6-diphosphate, 1.5 mM; KCl, 10 mM; and excess triosephosphate isomerase and α-glycerolphosphate dehydrogenase. For both 6-phosphofructokinase and aldolase, oxidation of NADH was followed at 340 nm.

Enzymes. Fructose-1,6-diphosphate aldolase, triosephosphate isomerase, α-glycerolphosphate dehydrogenase, citrate synthase, citrate lyase, phosphotransacetylase, lactate dehydrogenase, and malate dehydrogenase were purchased from Boehringer, Mannheim. Transcarboxylase was a gift from H. G. Wood.

Chemicals. Methylmalonyl-CoA was a gift from H. G. Wood. Oxaloacetate, NAD, NADH, NADP, PEP, acetyl-phosphate, and CoA were obtained form Boehringer, Mannheim, Germany. Fructose-6-phosphate, fructose-1,6-diphosphate, succinyl-CoA, acetylpyridine adenine dinucleotide, ATP, and pyruvate were obtained from Sigma Chemical Co., St. Louis, Mo. L-(−)-Lactate and D-(+)-lactate were purchased from Serva, Heidelberg, Germany. [1-^{14}C]-, [2-^{14}C]-, and [6-^{14}C]glucose and labeled lactate were obtained from Radiochemical Center, Amersham, England; [3-^{14}C]- and [3,4-^{14}C]glucose came from New England Nuclear, Boston, Mass.

RESULTS

Evidence for glucose fermentation via Embden-Meyerhof pathway. Results of fermentations of glucose or lactate labeled in different carbon atoms by cell suspensions of *B. fragilis*, under an atmosphere of 100% CO_2, can be seen in Table 1. When [3-^{14}C]glucose or [3,4-^{14}C]glucose were fermented, substantial amounts of label were found in the CO_2, while the specific activities of acetate and propionate were low. Fermentation of glucose labeled in carbon 1, 2, or 6 resulted in little labeling of CO_2, whereas the acid products were highly labeled. These results indicate that glucose is fermented via the Embden-Meyerhof pathway in this organism. This is further supported by the fact that fructose-1,6-diphosphate aldolase is present (Table 2), and extracts are capable of catalyzing the formation of fructose-1,6-diphosphate. However, this latter activity is not accomplished by the ATP-dependent fructose-6-phosphate kinase normally associated with the Embden-Meyerhof pathway. Instead, fructose-6-phosphate kinase is replaced by pyrophosphate:D-fructose-6-phosphate-1-phosphotransferase. "Replacement" of a conventional ATP-utilizing 6-phosphofructokinase with a pyrophosphate-dependent 6-phosphofructokinase is of considerable significance in that energy is conserved. In this regard it should be noted that the PP_i-dependent 6-phosphofructokinase is also present in *P. shermanii* (19).

Formation of succinate and propionate. Radioactive propionates obtained during the fermentation of glucose labeled in different carbons were degraded to determine in which carbon the label was located. The results of these degradations are shown in Table 3. As can be seen,

TABLE 1. *Fermentation of [^{14}C]glucose to propionate, acetate, and CO_2*[a]

Substrate	Glucose (cpm/mmol)	Propionate (cpm/mmol)	Acetate (cpm/mmol)	CO_2 (cpm/mmol)
[1-^{14}C]glucose	570,100	182,000	147,600	<500
[6-^{14}C]glucose	405,170	122,170	106,680	<500
[2-^{14}C]glucose	544,000	192,100	165,000	<500
[3-^{14}C]glucose	411,000	36,180	3,330	18,500
[3,4-^{14}C]glucose	460,300	39,420	4,660	24,800

[a] Because the fermentation was carried out under an atmosphere of 100% CO_2 with 0.5% bicarbonate in the incubation fluid, considerable dilution of labeled CO_2 occurred. The fermentations were carried out using unwashed cells, and this also caused some dilution of propionate and acetate. cpm, Counts per minute.

TABLE 2. *Glycolytic enzymes of B. fragilis*

Enzyme	Sp act[a]
6-Phosphofructokinase	<0.8
PP_i-dependent 6-phosphofructokinase	4,260
Fructose-1,6-diphosphate aldolase	1,280

[a] Specific activity is expressed in nanomoles per minute per milligram of protein.

TABLE 3. *Degradations of propionates from B. fragilis*

Substrate	Sp act of propionate[a]	Percent of ^{14}C as:		
		CH_3	CH_2	COOH
[1-^{14}C]glucose	186,629	49.2	48.1	2.7
[6-^{14}C]glucose	126,492	49.0	48.4	2.7
[2-^{14}C]glucose	182,850	47.2	49.3	3.4
[3-^{14}C]glucose	46,488	8.9	9.2	81.9
[3,4-^{14}C]glucose	49,626	—[b]	—[b]	86.4
[2-^{14}C]lactate + glucose	104,399	42.9	49.0	8.1
[2-^{14}C]lactate	132,210	45.3	51.1	3.6
[3-^{14}C]lactate + glucose	87,752	46.6	47.5	5.9
[3-^{14}C]lactate	102,926	47.3	51.3	1.4

[a] Counts per minute per millimole, obtained after total oxidation to CO_2. These figures differ somewhat from corresponding figures in Table 1 since different counting systems were used.

[b] This sample was lost after the carboxyl group had been obtained. It is obvious that almost all ^{14}C resides in the carboxyl group.

fermentation of glucose labeled in either 1, 2, or 6 gave propionate with label distributed equally between carbons 2 and 3. When lactate labeled in carbons 2 or 3 was fermented, either in the presence or absence of glucose, equal amounts of label were found in both the second and third carbons of propionate. It is clear that carbons 1, 2 and 5, 6 of glucose randomize completely during the formation of propionate, with randomization of carbons 2 and 3 of lactate also occurring. Therefore, the formation of propionate could not have occurred in *B. fragilis* via the nonrandomizing "acrylate" pathway, but rather is formed via a randomizing pathway similar to that found in either *P. shermanii* or *Veillonella*.

TABLE 4. *Specific activities of enzymes involved in the formation of oxaloacetate, succinate, and propionate in B. fragilis*

Enzyme	Sp act[a]
Transcarboxylase	<0.8
PEP carboxykinase (ADP)[b]	221.0
Pyruvate carboxylase	<2.0
PEP carboxyphosphotransferase	<0.8
Malate-lactate transhydrogenase	<0.8
Malate dehydrogenase	520.0
Fumarate reductase[c]	130.0
Methylmalonyl-CoA mutase[d]	+

[a] Specific activity as nanomoles per minute per milligram of protein.

[b] No activity was detected when IDP or GDP replaced ADP.

[c] See reference 15.

[d] See comments concerning enzyme assays in the text.

Enzyme assays (Table 4) reveal that formation of oxaloacetate in *B. fragilis* is by PEP carboxykinase, as is the case in *B. ruminicola* (25), and does not involve transcarboxylase as found in *P. shermanii* or pyruvate carboxylase as in *Veillonella*. Thus in *B. fragilis* the high energy of the phosphate bond in PEP is conserved in the form of ATP during the CO_2-dependent formation of oxaloacetate. The oxaloacetate is then reduced to malate by malate dehydrogenase (Table 4). Following dehydration of malate to fumarate, the latter is reduced to succinate by a membrane-bound fumarate reductase (Table 4). Since methylmalonyl-CoA mutase (Table 4) activity is detectable in *B. fragilis*, further metabolism of succinate to propionate is probably via methylmalonyl-CoA, which is assumed to be decarboxylated to propionyl-CoA as in *Veillonella*.

Formation of acetate. In that acetate is normally formed directly from pyruvate, glucose labeled in carbons 1 or 6 and in 2 should give rise to acetates labeled in the methyl or carboxyl carbons, respectively. Therefore, the results of the acetate degradations were surprising (Table 5). It is apparent that, during the fermentation of glucose to acetate, carbons 1, 2 and 5, 6 of

glucose randomized, but not completely. When lactate was the labeled substrate little randomization occurred, but the randomization increased in the presence of unlabeled glucose. Thus lactate must be metabolized to acetate via a nonrandomizing pathway, while most of the glucose must be metabolized to acetate by way of a randomizing pathway (i.e., via the symmetrical molecule fumarate).

TABLE 5. *Degradations of acetates from B. fragilis*

Substrate	Sp act of acetate[a]	Percent of ^{14}C as:	
		CH_3	COOH
[1-^{14}C]glucose	141,838	57.3	42.7
[6-^{14}C]glucose	108,890	56.3	43.7
[2-^{14}C]glucose	179,660	39.3	60.7
[3-^{14}C]glucose	5,953	—[b]	—[b]
[3,4-^{14}C]glucose	7,412	—[b]	—[b]
[2-^{14}C]lactate + glucose	205,728	8.0	92.0
[2-^{14}C]lactate	337,619	2.2	97.8
[3-^{14}C]lactate + glucose	247,294	92.9	7.1
[3-^{14}C]lactate	575,382	98.4	1.6

[a] Counts per minute per millimole, obtained after total oxidation to CO_2 (see footnote *a* of Table 3).

[b] Samples not degraded, since there was too little radioactivity.

TABLE 6. *Specific activities of enzymes involved in the formation of pyruvate in B. fragilis*

Enzyme	Sp act[a]
Pyruvate orthophosphate dikinase	<0.8
Pyruvate kinase	172.0[b]
Oxaloacetate decarboxylase	51.0
Malic enzyme (NADP)	48.6
Malic enzyme (NAD)	<0.8

[a] Specific activity as nanomoles per minute per milligram of protein.

[b] Reaction started by addition of ADP after 6 min of preincubation with PEP.

B. fragilis contains oxaloacetate decarboxylase and malic enzyme activities, in addition to pyruvate kinase activity (Table 6), and it is conceivable that when this organism is metabolizing glucose most of the pyruvate is formed from oxaloacetate or malate, using oxaloacetate decarboxylase and malic enzymes, respectively. If oxaloacetate and malate are in equilibrium with fumarate, then a randomization would occur (Fig. 1). However, a small portion of the pyruvate must be formed directly from PEP (via pyruvate kinase) in that the carbon atoms of acetate are not completely randomized. When lactate is fermented, the pathway to acetate is more direct. The lactate is simply oxidized to pyruvate via lactate dehydrogenase (Table 7), from which acetate is then formed. However, in the presence of glucose a pool of pyruvate may be formed, and some of it is shuffling back and forth to fumarate, leading to some randomization of the lactate carbons also.

DISCUSSION

It is clear from the results obtained from fermentation of differently labeled glucoses that *B. fragilis* ferments glucose via a pathway similar

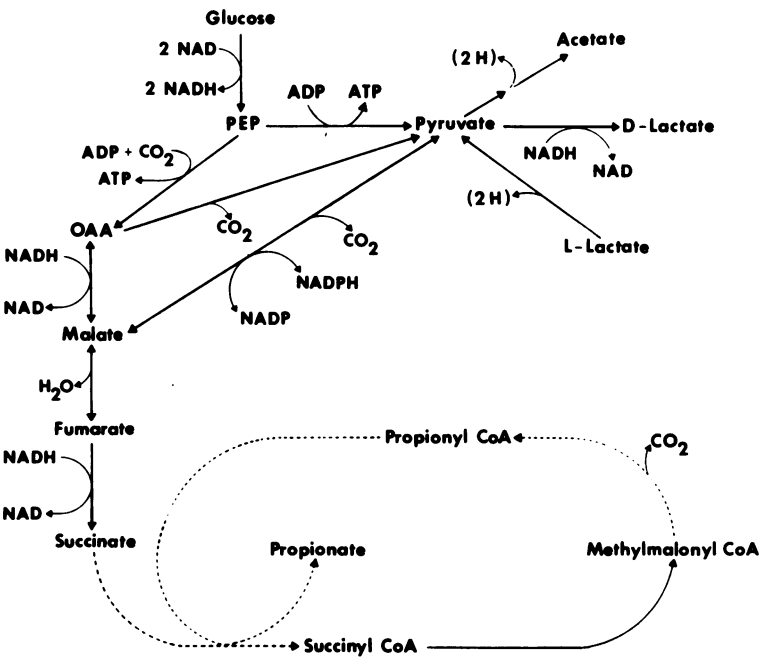

FIG. 1. *Probable pathway of succinate, propionate, and acetate formation in B. fragilis.*

to that of Embden-Meyerhof. Thus carbons 1, 2, and 6 clearly are converted to acetate and propionate, whereas carbons 3 and 4, to a large extent, yield CO_2 (Table 1). However, a substantial amount of carbons 3 and 4 is also recovered in propionate, and these carbons are not in complete isotopic equilibrium with CO_2.

Degradations of propionates formed during the fermentations of labeled glucose as well as of labeled lactate reveal that propionate must be formed via a pathway in which carbons 1 and 2 or 5 and 6 of glucose and carbons 2 and 3 of lactate randomize (Table 3). These results seem to exclude the "acrylate" pathway found in *B. ruminicola* (7, 32); instead they strongly support a pathway by which propionate is formed with fumarate and succinate as intermediates. These intermediates are symmetrical and allow randomization of the carbons. Such a pathway has been demonstrated in *Veillonella* (18) and *P. shermanii* (35) and has been extensively studied in these microorganisms.

A key enzyme of propionate formation in *P. shermanii* is transcarboxylase. This enzyme, which converts methylmalonyl-CoA to propionyl-CoA while carboxylating pyruvate to form oxaloacetate, appears to be absent in *B. fragilis*. This has led us to postulate that propionate formation in *B. fragilis* is similar to that in *Veillonella*. The results of the fermentation experiments and of enzyme analyses (Table 4) are compatible with the following reactions by which PEP is converted to propionate:

TABLE 7. *Specific activities of lactate dehydrogenases in B. fragilis*

Enzyme	Sp act[a]	
	Membrane	Soluble
D-(−)-Lactate dehydrogenase (NAD)	<0.8	244.0[b]
D-(−)-Lactate dehydrogenase (DCPIP[c])	<0.4	<0.4
L-(+)-Lactate dehydrogenase (DCPIP)	47.0	<1.4

[a] Specific activity as nanomoles per minute per milligram of protein.

[b] It was determined that this enzyme was a D-(−)-lactate dehydrogenase by assaying for the oxidation of D-(−)-lactate and L-(+)-lactate to pyruvate with acetylpyridine adenine dinucleotide as electron acceptor; see the text (34).

[c] DCPIP, 2,6-Dichlorophenolindophenol.

marate reductase activity is lacking; thus the above pathway could not function.

It is interesting that transcarboxylase, pyruvate carboxylase, and PEP carboxyphosphotransferase are not used by *B. fragilis* to form oxaloacetate. Instead the organism possesses a PEP carboxykinase, which, while carboxylating PEP to oxaloacetate, conserves the high-energy phosphate of PEP in the form of ATP (reaction 1) (Table 3). The use by *B. fragilis* of PEP carboxykinase for the synthesis of oxaloacetate, as well as the presence of the remainder of the pathway to succinate and propionate that involves possible ATP synthesis during reduction of fumarate, explains why growth of *B. fragilis* is CO_2 dependent.

$$\text{PEP} + \text{ADP} + CO_2 \xrightarrow{\text{PEP carboxykinase}} \text{oxaloacetate} + \text{ATP} \quad (1)$$

$$\text{oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{malate dehydrogenase}} \text{malate} + NAD^+ \quad (2)$$

$$\text{malate} \xrightarrow{\text{fumarate hydratase}} \text{fumarate} + H_2O \quad (3)$$

$$\text{fumarate} + \text{NADH} + H^+ \xrightarrow{\text{fumarate reductase}} \text{succinate} + NAD^+ \quad (4)$$

$$\text{succinate} + \text{propionyl-CoA} \xrightarrow{\text{acyl-CoA transferase}} \text{succinyl-CoA} + \text{propionate} \quad (5)$$

$$\text{succinyl-CoA} \xrightarrow{\text{methylmalonyl-CoA mutase}} \textit{R}\text{-methylmalonyl-CoA} \quad (6)$$

$$\textit{R}\text{-methylmalonyl-CoA} \xrightarrow{\text{methylmalonyl-CoA racemase}} \textit{S}\text{-methylmalonyl-CoA} \quad (7)$$

$$\textit{S}\text{-methylmalonyl-CoA} \xrightarrow{\text{methylmalonyl-CoA decarboxylase}} \text{propionyl-CoA} + CO_2 \quad (8)$$

$$\text{PEP} + \text{ADP} + 2\text{NADH} + 2H^+ \rightarrow \text{propionate} + \text{ATP} + 2NAD^+ + H_2O \quad \text{(sum)}$$

These reactions also explain earlier results (15) which showed that propionate formation is hemin dependent. In the absence of hemin, fu-

The results of the acetate degradations (Table 5) show that when glucose is fermented to acetate there is a randomization of carbons 1, 2 and 5, 6 of glucose, although it is not complete. In addition, while little randomization occurred when lactate was fermented, the presence of unlabeled glucose increased this randomization. These results can be explained by considering the reaction outlined in Fig. 1, which is based on enzyme and tracer studies presented in this communication. Glucose is fermented to PEP, which is converted either to oxaloacetate or directly to pyruvate. Oxaloacetate and malate are in equilibrium with fumarate, at which level randomization of the carbons occurs. It is conceivable, especially in the presence of high concentrations of CO_2 (bicarbonate), that the major part of PEP is converted to oxaloacetate and malate. Pyruvate would then be formed by decarboxylation either of oxaloacetate or of malate as catalyzed by oxaloacetate decarboxylase or the malic enzyme, respectively. Acetate produced from this pyruvate would be labeled in both carbons when the fermentation was with [1-^{14}C]-, [2-^{14}C]-, or [6-^{14}C]glucose. Pyruvate formed directly from PEP would, on the other hand, be labeled only in a single carbon; and acetate formed from [1-^{14}C]- or [6-^{14}C]glucose would be methyl labeled, whereas [2-^{14}C]glucose would yield carboxyl-labeled acetate. The somewhat higher randomization of the lactate carbons in the presence of glucose may simply occur because pyruvate is in partial equilibrium with oxaloacetate, malate, and fumarate. The pool of these acids may be larger during the glucose-lactate fermentation than when lactate alone is fermented.

It should be mentioned that earlier experiments with *P. shermanii* also showed a randomization of carbon 1 of glucose when it was fermented to acetate. In fact, when incubated under 100% CO_2, there was a more extensive randomization than encountered with *B. fragilis*; almost equivalent amounts of label were found in both carbons and acetate (38). Incubation of *P. shermanii* in an atmosphere of N_2 resulted in less randomization (40). When lactate was fermented to acetate, little randomization occurred (13). The authors did not explain this randomization, but it is conceivable that it might have occurred in a manner similar to that which we have postulated for *B. fragilis*. If this is the case, the findings with *P. shermanii* support the pathway proposed above for acetate formation in *B. fragilis* (Fig. 1).

When considering the pathways involved in the catabolism of glucose to succinate, propionate, and acetate, it becomes clear that *B. fragilis* has evolved a set of enzymes that allow it to gain the maximum energy possible from this substrate. For instance, rather than expend an ATP to form fructose-1,6-diphosphate via a conventional 6-phosphofructokinase, this organism uses inorganic pyrophosphate. The enzyme that carries out this reaction, pyrophosphate:D-fructose-6-phosphate-1-phosphotransferase (PP_i-dependent 6-phosphofructokinase) was first discovered in *Entamoeba histolytica* by Reeves et al. in 1974 (21). Since then it has also been found in *P. shermanii* (19), in marine species of *Alcaligenes* (24), and in *Pseudomonas marina* (24). (See review by Wood et al. [39].)

A second energy-conserving enzyme found in *B. fragilis* is PEP carboxykinase, which catalyzes the carboxylation of PEP to oxaloacetate with the concomitant formation of ATP. Except for transcarboxylase, any other enzyme that carboxylates either PEP or pyruvate to oxaloacetate would result in the net loss of an ATP.

Finally, if ATP is indeed generated via a primitive type of electron transport system involving membrane-bound cytochrome *b* and fumarate reductase (15), this would also add considerably to the numbers of ATPs formed during the fermentation of glucose. Taking all of the above energy-saving and -generating reactions into consideration, as well as the amounts of the various fermentation products made (15), it is possible to calculate that the maximum moles of ATP that might be gained during the catabolism of a mole of glucose is 4.56 (assuming that the reduction of a mole of fumarate to succinate results in 2 mol of ATP). This value is in very good agreement with the experimental values of 4.48 and 5.05 ATPs per mol of glucose fermented, as determined from yield experiments; it was assumed that Y_{ATP} = 10.5 g of cells per mol of ATP (15).

Thus it would seem that by evolving such a CO_2-dependent metabolism, *B. fragilis* has found a way to compete successfully with both the human gut and the other microorganisms that live with it there.

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