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Influence of CO_2-HCO_3^- Levels and pH on Growth, Succinate Production, and Enzyme Activities of *Anaerobiospirillum succiniciproducens*

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Growth and succinate versus lactate production from glucose by *Anaerobiospirillum succiniciproducens* was regulated by the level of available carbon dioxide and culture pH. At pH 7.2, the generation time was almost doubled and extensive amounts of lactate were formed in comparison with growth at pH 6.2. The succinate yield and the yield of ATP per mole of glucose were significantly enhanced under excess-CO_2-HCO_3^- growth conditions and suggest that there exists a threshold level of CO_2 for enhanced succinate production in *A. succiniciproducens*. Glucose was metabolized via the Embden-Meyerhof-Parnas route, and phosphoenolpyruvate carboxykinase levels increased while lactate dehydrogenase and alcohol dehydrogenase levels decreased under excess-CO_2-HCO_3^- growth conditions. Kinetic analysis of succinate and lactate formation in continuous culture indicated that the growth rate-linked production rate coefficient (K) cells was much higher for succinate (7.2 versus 1.0 g/g of cells per h) while the non-growth-rate-related formation rate coefficient (K′) was higher for lactate (1.1 versus 0.3 g/g of cells per h). The data indicate that *A. succiniciproducens*, unlike other succinate-producing anaerobes which also form propionate, can grow rapidly and form high final yields of succinate at pH 6.2 and with excess CO_2-HCO_3^- as a consequence of regulating electron sink metabolism.

Anaerobic bacteria can produce a variety of organic acids as end products (e.g., lactic, acetic, butyric, caproic, propionic, succinic, and formic acids) of glucose metabolism (27). The metabolism of anaerobic species which produce high yields of lactic and propionic acids have been well studied (26) and are used for commercial production of foods and chemicals (11). Recently, the fermentative production of succinic acid from carbohydrates by anaerobic bacteria has become of applied significance as a renewable-resource-based route for C_4 chemical feedstocks used in oxychemical manufacture (11). Also, new physiological studies on these kinds of anaerobic bacteria indicate that their high growth yield is associated with generation of a membrane potential upon succinate efflux (17).

Although many different kinds of bacteria (e.g., *Escherichia coli*, *Bacteroides fragilis*, and *Wolinella succinogenes*, etc.) produce succinate as an anaerobic end product, few species that make it as the major end product in high concentration are reported (8, 13, 23, 24, 26, 27). In gastrointestinal tracts such as the rumen, high levels of CO_2 are formed and the natural microbial flora (e.g., *Ruminococcus flavofaciens* and *Bacteroides succinogenes*) produces succinic acid as a major end product of carbohydrate degradation (8, 9, 24).

All bacteria require carbon dioxide for anabolism. If CO_2 is limiting in bacterial culture medium and the level is less than that needed for effective biosynthesis of cell matter, then cell growth is affected (4, 12, 19, 20). In anaerobic bacteria that produce succinate, CO_2 is used both as a catabolic substrate and for cell synthesis. In these kinds of anaerobes, fumarate reduction to succinate is coupled to electron transport-mediated phosphorylation (13).

Recently, we have begun studies on the fermentation of carbohydrate feedstocks into succinate with *Anaerobiospirillum succiniciproducens* as the model organism. In the presence of CO_2, this organism makes high concentrations of succinate which precipitates out of the fermentation medium as a calcium salt. Little has been reported on the physiology of *A. succiniciproducens* other than general growth and metabolic properties used in the original taxonomic description of this nongastrointestinal species (3). In the present communication we report on (i) the catabolic pathway for glucose fermentation in *A. succiniciproducens*, (ii) the influence of carbon dioxide and pH on growth, fermentation kinetics, and carbon balance and the profile of products formed; (iii) the changes in the levels of key catabolic enzymes with respect to pH and available carbon dioxide; and, (iv) the relationship between specific rates of growth and product formation under various cultivation conditions in continuous culture.

MATERIALS AND METHODS

Chemicals. All chemicals used were of reagent grade and were obtained from either Mallinckrodt, Paris, Ky., or Sigma Chemical Co., St. Louis, Mo. Gases were supplied by Linde, Michigan Welding, East Lansing, Mich., and were scrubbed free of oxygen by passage over heated (370°C) copper filings.

Organism and growth conditions. *A. succiniciproducens*

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(ATCC 29305) was transferred in general culture medium containing the following (in g/liter of distilled water): glucose, 20; corn steep liquor (Sigma; 50% solids), 10; K_2HPO_4, 3; NaCl, 1; $(NH_4)_2SO_4$, 1; and Na_2CO_3, 5. The medium was heat sterilized (20 min at 121°C) in vials sealed with butyl rubber bungs and with a nitrogen headspace. To the sterile medium (100 ml) in 158-ml vials, 10 M H_2SO_4 was added to reduce the pH to 6.8 ± 0.1. The nitrogen headspace was replaced by carbon dioxide, and $Na_2S \cdot 9H_2O$ (final concentration, 0.025%) was added in order to establish strict anaerobic conditions. After 15 min, the reduced medium was inoculated and the vials were incubated in a rotary shaker (at 37°C). Media were inoculated with 5% (vol/vol) samples of cultures grown overnight.

The following components were supplied to fermentor cultures grown in pH-controlled vessels (in g/liter): corn steep liquor (50% solids), 20; K_2HPO_4, 1.5; $MgCl_2 \cdot 6H_2O$, 0.2; and tryptophan, 0.02. Glucose was sterilized separately. Carbon dioxide was supplied as CO_2 gas, Na_2CO_3, magnesium carbonate, $CaCO_3$, or a combination of these, without there being any significant influence of CO_2 source on growth. Kimax jars (4-liter) (Baxter Scientific Products, Romulus, Mich.) containing 3 liters of medium were used, the jars were equipped with a pH probe, and the culture was mixed by placing the jars on a magnetic stirrer.

Ten-liter batch fermentations for preparation of enzyme extracts were performed in stirred pH-controlled 16-liter glass carboys. Continuous fermentation cultures with a 330-ml working volume were performed in a Biflo (New Brunswick) model C30 fermentor. The CO_2 was supplied by gassing with O_2-scrubbed CO_2 and by the addition of Na_2CO_3. Prior to inoculation, strict anaerobic conditions were established with 0.025% $Na_2S \cdot 9H_2O$ and the initial pH was adjusted to 6.7 ± 0.1 (unless stated otherwise) with H_2SO_4. Throughout the fermentation the pH was controlled at 6.2 ± 0.1 with 3 M NaOH.

Analytical. Glucose and fermentation products were analyzed by high-performance liquid chromatography in acidified (1% concentrated HCl) samples as previously described (5). The metabolites were eluted with 0.012 N H_2SO_4 from a cation-exchange resin in the hydrogen form. They were detected with a differential refractometer, recorded, and quantified with a Waters 840 integrator. The concentration was computed from the area under the curve. Separations were performed on a Bio-Rad HPX-87 column (300 by 7.8 mm [internal diameter]). The level of succinic acid was also determined by an enzymatic assay using a commercial kit from Boehringer Mannheim Biochemical.

Yields of fermentation products were calculated as a percentage of the glucose consumed and corrected in accordance with the dilution factor of base (NaOH and Na_2CO_3) addition through the pH control system. Carbon balance and recovery of total available hydrogen (electron balance) were calculated by standard methods previously described (16). Dry cell mass was computed from a curve relating optical density at 660 nm (OD_{600}) to dry weight as previously described (16), and 1.0 OD represented 330 ± 15 mg (dry weight) per liter.

Preparation of cell extracts. Cultures were harvested during the late exponential phase of growth with a Pellicon tangential-flow membrane system (Millipore Corp., Bedford, Mass.) fitted with a 100,000-molecular-weight cutoff membrane. The system was made anaerobic by flushing with sulfide-reduced distilled water and N_2 gas. The concentrated cells were pumped into a 250-ml polycarbonate centrifuge bottle which had been flushed with N_2. The cells were centrifuged at 8,000 × *g* for 15 min and washed twice with distilled water containing 2 mM dithiothreitol (DTT).

Cell extracts were prepared by passage through a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) at 20,000 lb/in^2. The French pressure cell was made anaerobic with N_2 for 20 min prior to the addition of cells. The disrupted cells were collected in an anaerobic tube and centrifuged at 20,000 × *g*. The supernatant was stored under N_2 in rubber-bunged vials at −70°C until used. Protein was measured by the Lowry method (15), with bovine serum albumin as the standard.

Enzyme assays. All enzyme assays were performed by strict anaerobic techniques described previously (28). Glass cuvettes (1.7-ml total volume) were sealed with grey stoppers and made anaerobic by repeatedly evacuating and flushing with N_2 gas. All buffers and substrates were prepared in glass vials sealed with rubber stoppers and rendered anaerobic by the above procedure. All additions to the cuvettes were made with a microliter syringe, to give a final liquid volume of 1 ml. Enzyme specific activities were calculated from the linear part of the reaction, and values for activity were determined from a minimum of three separate measurements each on two different cell extract preparations. All assays were performed at 37°C in a Cary 219 spectrophotometer. The wavelengths and millimolar extinction coefficients for NAD, NADH, NADP, and NADPH were 340 nm and 6.22 cm^{-1} mM^{-1}, respectively, and for methyl viologen they were 578 nm and 9.78 cm^{-1} mM^{-1}, respectively. The wavelength and extinction coefficient for methylene blue were 668 nm and 6.3 cm^{-1} mM^{-1}, respectively, and for benzyl viologen were 578 nm and 8.65 cm^{-1} mM^{-1}.

Hexokinase (EC 2.7.1.1) was assayed by monitoring the reduction of NADP at 340 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 7.5) buffer, 60 mM $MgCl_2$, 1 mM DTT, 0.5 mM NADP, 2 mM ATP, 15 mM glucose, cell extract, and 2 U of glucose-6-phosphate dehydrogenase. Fructokinase (EC 2.7.1.11) was measured at 340 nm in a reaction mixture containing 0.1 M Tris-HCl (pH 7.8), 2 mM ATP, 5 mM $MgSO_4$, 2 mM fructose-6-phosphate, 0.3 mM NADH, 50 mM KCl, 1 mM DTT, 2 U of aldolase and triose-phosphate-isomerase, 10 U of glycerophosphate dehydrogenase, and cell extract. The reaction was initiated by the addition of fructose-6-phosphate. Fructose-1,6-biphosphate aldolase (EC 4.1.2.13) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) activities were measured as described previously (28). Pyruvate kinase (EC 2.7.1.40) was measured in a reaction mixture containing 0.1 M Tris-HCl buffer (pH 7.5), 8 mM $MgCl_2$, 80 mM KCl, 5 mM phosphoenolpyruvate (PEP), 10 mM ADP, 2 mM fructose-1,6-diphosphate, 10 U of lactate dehydrogenase, 0.3 mM NADH, and cell extract. PEP-carboxykinase (EC 4.1.1.49) activity was monitored in a reaction mixture containing 50 mM sodium hydrogen maleate buffer (pH 5.5), 10 mM $MgCl_2$, 10 mM ADP, 25 mM $NaHCO_3$, 20 mM PEP, 0.3 mM NADH, cell extract, and 2 U of malate dehydrogenase. Pyruvate-ferredoxin oxidoreductase (EC 1.2.7.1) and L-lactate dehydrogenase (EC 1.1.1.27) activities were measured as described previously (14). Alcohol dehydrogenase (EC 1.1.1.1) was measured with the following reaction mixture: 0.1 M Tris-HCl buffer (pH 7.8), 10 mM acetaldehyde, 0.3 mM NADH, 2 mM DTT, and cell extract. Hydrogen-oxidizing hydrogenase activity (EC 1.12.1.2) was measured by monitoring the reduction of methyl viologen, benzyl viologen, methylene blue, NAD, or NADP. The assay system contained 0.1 M Tris-HCl buffer (pH 7.8), 2 mM

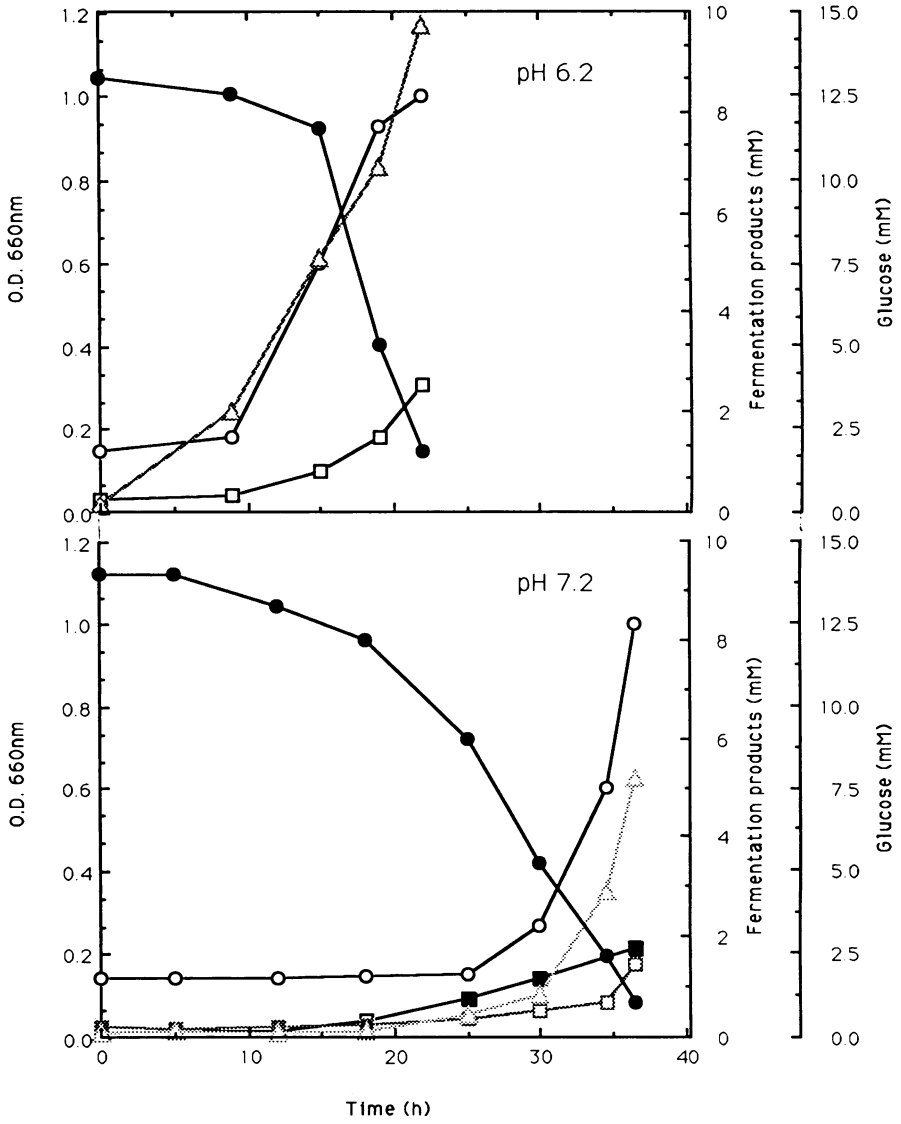

FIG. 1. Effect of pH on growth and substrate-product transformation kinetics during glucose fermentation by *A. succiniciproducens*. Cells were grown to OD = 1.0, (representing late exponential phase) in a 10-liter fermentor under constant pH control, and the carbon dioxide was supplied in excess with 4.7 g of Na_2CO_3 per liter and by continuous gassing with CO_2 (1 liter/min). The inoculum was grown at the corresponding pH and CO_2-HCO_3^- concentration used in the 10-liter fermentor. Symbols: ○, OD; ●, glucose; △, succinate; □, acetate; ■, lactate.

electron acceptor, sodium dithionite or DTT, cell extract, and a headspace of hydrogen. Formate dehydrogenase (EC 1.2.1.2) was measured as described for hydrogenase, except that the buffer was 50 mM phosphate buffer (pH 7.2) and 5 mM sodium formate replaced hydrogen. Acetate kinase (EC 2.7.2.1) activity was measured as described previously (14). Malate dehydrogenase (EC 1.1.1.38) reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.1), 5 mM oxaloacetate, 0.3 mM NADH, 2 mM DTT, and cell extract. Fumarate reductase (EC 1.3.1.6) activity was measured by monitoring NADH oxidation. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 7.2), 5 mM fumarate, 0.3 mM NADH, 2 mM DTT, and cell extract. Methylmalonyl coenzyme A carboxyltransferase (EC 2.1.3.1) was measured as described elsewhere (10).

RESULTS

Glucose fermentation parameters. Figure 1 compares the time course for growth and end product formation during glucose fermentation in excess CO_2-HCO_3^- by *A. succiniciproducens* at pH 6.2 versus 7.2. Cells were grown in a batch fermentor to an OD_{660} of 1 (representing late exponential phase) at constant pH. The glucose fermentation at pH 7.2 was associated with a long lag phase (20 to 25 h) for growth that was not observed at pH 6.2. At a fermentation pH of 6.2, growth and succinate production were directly related to glucose consumption and lactate was formed in trace amounts; but at a pH of 7.2, lactic acid was a significant end product. Acetate was formed under both pH conditions, and small amounts of ethanol (less than 2 mM) were formed

TABLE 1. Influence of carbon dioxide on the glucose fermentation balance of *A. succiniciproducens*[a]

End products recovered[b]	Fermentation balance (mol of product/100 mol of glucose consumed) at CO_2 ration[c] of:		
	98	49	6.5
Cells[d]	113	105	44
Succinate	121	111	43
Acetate	67	68	16
Formate	12	11	12
Lactate	ND	ND	87
Ethanol	ND	ND	9
Total carbon	743	696	539
Carbon recovered	1.05	1.07	0.89
Total H	2,278	2,142	1,930
Electron recovered	1.05	1.12	1.24
ATP yield	2.55	2.47	0.75
Y_{ATP} (g of cells/mol of ATP)	11.9	11.4	11.8

[a] Cells were grown in 158-ml serum vials on 20 g of glucose per liter. The pH was adjusted to 6.2 ± 0.2 with 4 N NaOH.
[b] Determined following the complete dissolution of magnesium carbonate.
[c] Carbon dioxide was supplied with various amounts of added magnesium carbonate which dissolved during the fermentation. The numbers represent the initial ratio (moles of CO_2 per 100 moles of glucose). Values are means from four replicates.
[d] Cell carbon was calculated with $CH_2O_{0.5}N_{0.21}$ (24).

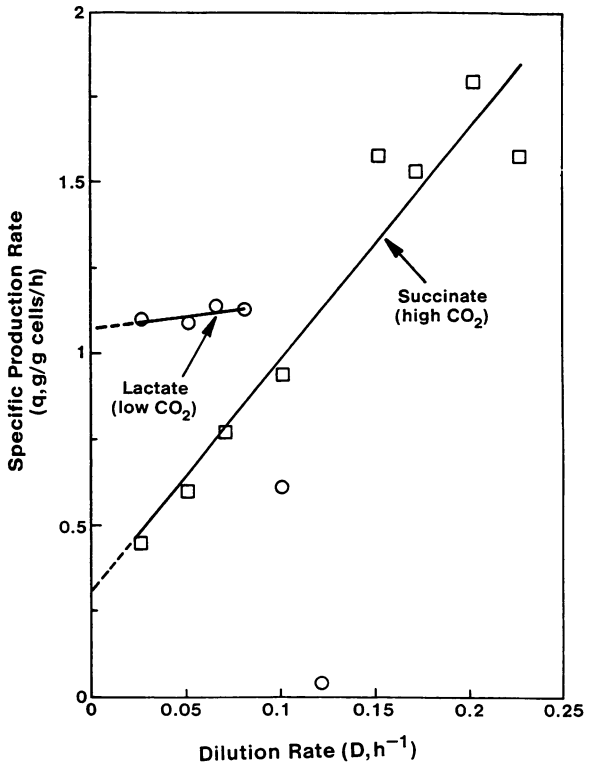

FIG. 2. Influence of CO_2-HCO_3^- levels on lactate versus succinate production by *A. succiniciproducens* during continuous culture fermentations at various dilution rates. Means of duplicate chemostat runs were computed from steady-state data and plotted as the best-fit line ($q = KD + K'$) as previously described (22). The volume of the chemostat culture was 330 ml. For succinate production, the feed medium contained 4.7 g of Na_2CO_2 per liter and the vessel was gassed with 35 ml of CO_2 per min. For lactate production, the medium contained 1.10 g of Na_2CO_2 per liter and the vessel was not gassed.

at the higher pH. Cell-doubling times of 22.2 and 3.9 h and maximal specific growth rates (μ_{max}) of 0.31 and 0.17 h^{-1} were calculated for pH 6.2 and 7.2 fermentations, respectively.

The effect of carbon dioxide levels on the glucose fermentation balance in cells grown at pH 6.2 is presented in Table 1. Carbon dioxide was supplied as solid magnesium carbonate which generated CO_2-HCO_3^- during the fermentation and meant that accurate determination of total CO_2 consumption during growth was not possible. Lower levels of CO_2 would not support growth of *A. succiniciproducens*. When the molar ratio of CO_2-HCO_3^- to glucose was between 1.0 and 0.5, about 15% of the available carbon (from glucose plus CO_2-HCO_3^-) was incorporated into cell mass. Under these growth conditions, 90% of the glucose was consumed and 65% of the carbon was converted to succinate. When the molar ratio of CO_2-HCO_3^- to glucose was reduced to 0.065, 45 to 60% of the glucose was consumed and only 8% of the carbon was converted into cell mass. Approximately 50% of the carbon was fermented into lactate, 30% was converted into succinate, and the balance was detected in ethanol, formate, and acetate. The ratio between succinate and acetate produced under low CO_2-HCO_3^- levels (CO_2-HCO_3^-–glucose ratio = 0.065) was 60% higher than that found at elevated carbon dioxide levels. The fermentation balances indicated that nearly 100% of the glucose metabolized was recovered in the end products. Lactate and ethanol were not formed at elevated CO_2-HCO_3^- concentrations. The molar amount of ATP formed per mole of metabolized glucose and the molar growth yield on ATP (Y_{ATP}) were estimated with the fermentation balances found under different growth conditions presented in Table 1. Under low CO_2-HCO_3^- concentrations, the ATP yield was 0.75 mol of ATP per mol of glucose, whereas under medium and high CO_2-HCO_3^- concentrations, the ATP yield was 2.55 and 2.47, respectively. Although ATP production was greatly affected by the level of available CO_2-HCO_3^-, the Y_{ATP} for *A. succiniciproducens* of 11.4 to 11.8 g of cells per mol of ATP is similar to the widely accepted value of 10.5 g of cells per mol of ATP calculated for many bacterial species (25).

Continuous culture fermentations were conducted in order to assess steady-state succinate and lactate production rates at high and low CO_2-HCO_3^- levels. Figure 2 compares the relationship between the specific rates of product formation and growth as they were computed from steady-state process data. The growth rate-associated production rate coefficients (K) for succinate and lactate were 7.2 and 1.0 g of product per g of cells per h, respectively. The non-growth-rate-linked coefficients (K', g of product per g of cells per h) were 0.3 for succinate and 1.1 for lactate. The growth rate-linked succinate formation rate coefficient (K = 7.2 g of succinate per g of cells per h) was 24 times higher than the non-growth-rate-related coefficient (K' = 0.3 g of succinate per g of cells per h). Under optimal conditions for lactate production, these two rate coefficients have almost the same value. The ratio between the growth rate-associated production coefficients of succinate and lactate was 7.2 (K for succinate/K for lactate) while the ratio of the non-growth-rate-related coefficients was 0.3 (K' for succinate/K' for lactate), indicating that optimal conditions for growth were closely related to succinate and not lactate production.

Catabolic enzyme activities. Key enzymes participating in

TABLE 2. Influence of pH and CO_2-HCO_3^- on enzyme levels in *A. succiniciproducens*[a]

Enzyme	Sp act (nmol/mg of protein/min) at:	
	pH 7.2 (low CO_2-HCO_3^-)[b]	pH 6.2 (high CO_2-HCO_3^-)[c]
PEP-carboxykinase	10	356
Pyruvate-ferredoxin oxidoreductase	123	74
Malate dehydrogenase	18,800	20,900
Fumarate reductase	380	118
Pyruvate kinase	9	8
Acetate kinase	1,370	3,320
Lactate dehydrogenase	27	ND
Alcohol dehydrogenase	44	ND

[a] Extracts were prepared from fermentor cells grown at a constant pH with an initial glucose concentration of 10 g/liter and harvested in the late exponential phase of growth.

[b] Carbon dioxide was supplied with Na_2CO_3 (1.6 g/liter).

[c] Carbon dioxide was supplied in excess with Na_2CO_3 (4.7 g/liter) and continuous gassing with (1 liter of CO_2 per min). ND, not detectable (<0.2 nmol/mg of protein per min).

the metabolism of glucose to PEP, including hexokinase and glyceraldehyde-3-phosphate dehydrogenase, were measured in cell extracts. Fructokinase, which participates specifically in the Embden-Meyerhof-Parnas route, and fructose-1,6-biphosphate aldolase were also detected.

Table 2 compares the influence of pH and CO_2-HCO_3^- on the levels of fermentative enzyme activities responsible for end product formation. These growth conditions were chosen in order to clearly distinguish the changes in carbon and electron flow to products. Under low pH and high CO_2-HCO_3^- concentrations, the major product was succinate, whereas at pH 7.2 and low CO_2-HCO_3^- concentrations, the highest levels of lactate, ethanol, and acetate were observed. In cells grown at pH 6.2 and high CO_2 concentrations, PEP-carboxykinase was increased 35-fold and lactate dehydrogenase and alcohol dehydrogenase were not detected. Low levels of pyruvate kinase and high levels of malate dehydrogenase, fumarate reductase, and acetate kinase were found in cell extracts from cells grown under either condition. Cell extract lacked detectable levels of formate dehydrogenase and methylmalonyl coenzyme A transcarboxylase activity. Lactate dehydrogenase activity did not require fructose-1,6-diphosphate as an activator.

DISCUSSION

In general, these data provide a biochemical explanation for the route of glucose fermentation and for the high growth rate and succinate yield of *A. succiniciproducens* at high CO_2-HCO_3^- levels and pH 6.2. The influence of high CO_2-HCO_3^- levels on key catabolic enzyme levels is consistent with the observed glucose fermentation product ratios and the calculated ATP yield. The influence of CO_2-HCO_3^- on product formation in *A. succiniciproducens* differs, however, from that reported in gastrointestinal anaerobes in that these organisms produce propionate in addition to succinate (2). The findings here suggest that in *A. succiniciproducens* there is a threshold level of CO_2-HCO_3^- required for succinate production in favor of other end products, irrespective of the growth pH.

Figure 3 illustrates the proposed glucose fermentation pathway for *A. succiniciproducens* based on end products formed and enzyme activity at pH 6.2 and high CO_2-HCO_3^- levels versus pH 7.2 and low CO_2-HCO_3^- levels. The described enzyme activities and radiotracer studies (not shown here) provide evidence that *A. succiniciproducens*, like other succinate-producing anaerobes described elsewhere (27), uses the Embden-Meyerhof-Parnas pathway for glycolysis. Fermentation results indicate that lactate production in *A. succiniciproducens* is controlled by high pH and succinate production is controlled by CO_2 availability. At pH 6.2 and high CO_2-HCO_3^- levels, succinate is the major product, PEP-carboxykinase levels are high, and neither lactate dehydrogenase nor ethanol dehydrogenase is detectable, whereas at pH 7.2 and low CO_2-HCO_3^- levels, PEP-carboxykinase levels are lower and lactate dehydrogenase and alcohol dehydrogenase are present.

Figure 4 compares the flux of carbon through the metabolic pathways of *A. succiniciproducens* grown under excess versus limited carbon dioxide. The glucose fermentation route described here indicates the following energy conservation steps, excluding transport-coupled phosphorylation coupled to succinate efflux, in *A. succiniciproducens*: 2 mol of ATP via the Embden-Myerhof-Parnas pathway of glycolysis, 1 mol of ATP per mol of fumarate via electron transport phosphorylation (ETP), and 1 mol of ATP per mol of acetate via substrate-level phosphorylation (SLP). Cell growth yields were directly correlated to the level of succinate and acetate formed. The ratio of glucose carbon used for cell mass production to that used for succinate and acetate formation {C(cells)/[C(succinate) + C(acetate)]} was similar (0.18 to 0.20) at different CO_2-HCO_3^- levels. Under conditions of excess CO_2-HCO_3^-, carbon flux through the energy-yielding routes (ETP plus SLP) was 2.2 times higher and cell yield was nearly twofold higher. Under carbon dioxide limitation, only 30% of the carbon flux was diverted through SLP [SLP(low CO_2-HCO_3^-)/SLP(high CO_2-HCO_3^-) = 0.32] and less than 50% was directed through ETP [ETP(low CO_2-HCO_3^-)/ETP(high CO_2-HCO_3^-) = 0.49] compared with results under excess-CO_2-HCO_3^- growth conditions. A comparison of the carbon flux through PEP-carboxykinase with carbon flux via pyruvate kinase indicates that six times more glucose carbon is directed to succinate formation under conditions of excess CO_2-HCO_3^- (0.651/0.197 = 3.3 versus 0.319/0.6 = 0.53) and is in accordance with the much higher levels of PEP-carboxykinase found under these growth conditions.

The effect of carbon dioxide level on the generation time and growth rate of *A. succiniciproducens* is similar to that reported for *B. fragilis* (2). In both organisms the attainable growth rate decreased by almost 50% under CO_2 limitation, the lag growth phase increased, and longer generation times were inversely related to the concentration of CO_2. In *B. fragilis* the maximal cell yield on glucose was increased by more than 14% when the organism was grown under 100% N_2 instead of 100% CO_2 (2), while in *A. succiniciproducens* and *Bacteroides ruminicola* (10) low available-CO_2 concentrations were associated with a decrease in cell mass yield. *B. fragilis*, unlike *A. succiniciproducens*, contains a methylmalonyl transcarboxylase for conversion of succinate to propionate and CO_2. Under 100% N_2, *B. fragilis* produces more propionate, at low specific growth rates a near homopropionate fermentation was achieved (propionate/succinate = 16), and increases in this activity as well as PEP-carboxykinase levels under CO_2 limitation occur (2). *A. succiniciproducens* neither produces propionate nor possesses a methylmalonyl transcarboxylase activity, and the organism is dependent on the supply of external CO_2 and

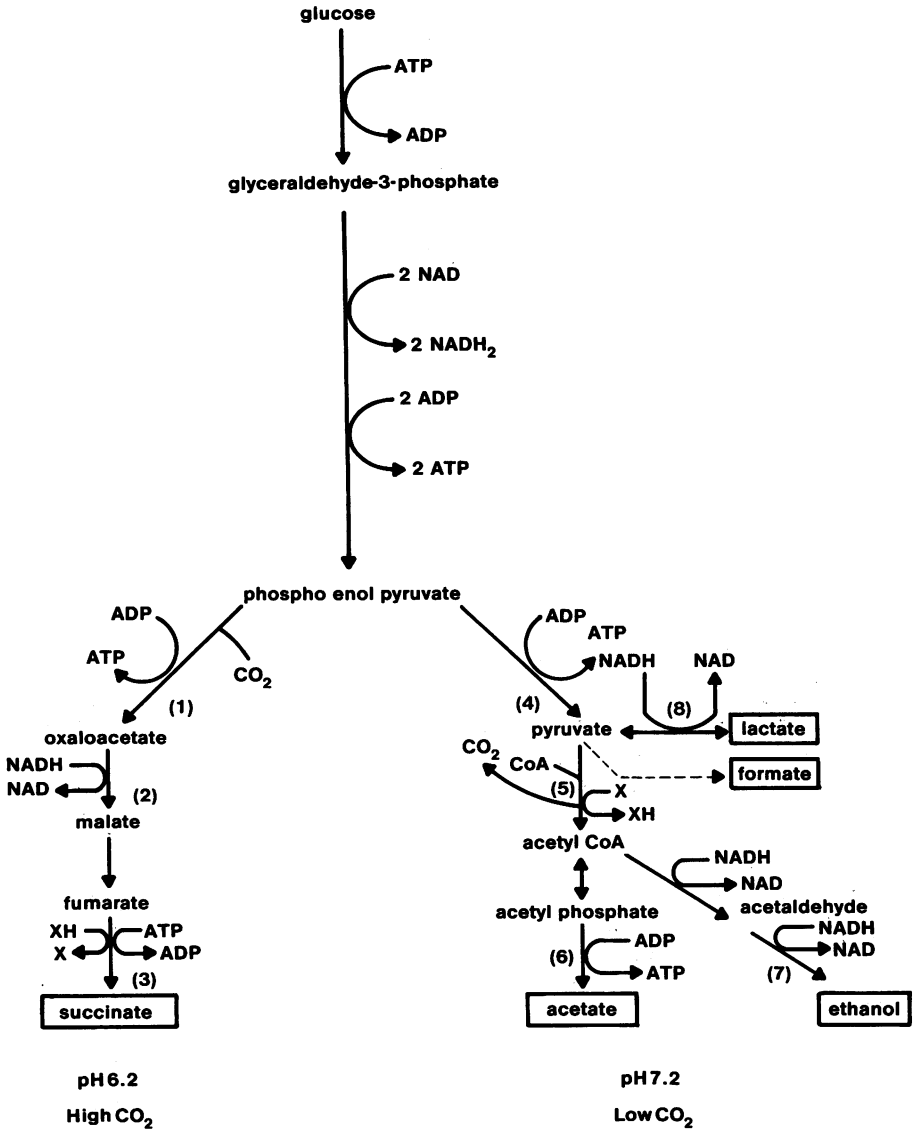

FIG. 3. Proposed catabolic pathway for glucose fermentation in *A. succiniciproducens*. Steps: 1, PEP-carboxykinase; 2, malate dehydrogenase; 3, fumarate reductase; 4, pyruvate kinase; 5, pyruvate-ferredoxin oxidoreductase; 6, acetate kinase; 7, alcohol dehydrogenase; 8, lactate dehydrogenase.

limited CO_2 generated from the pyruvate dehydrogenase reaction.

Recently, the influence of pH on the regulation of growth and the formation of catabolic end products has been examined in obligate anaerobes (1, 6, 7, 18). In *Clostridium acetobutylicum*, acid pH causes a shift from acetate and butyrate production to acetone, butanol, and ethanol production (7). In *Sarcina ventriculi*, a shift to lower pH altered pyruvate metabolism from hydrogen acetate formation to ethanol production (6). In *Lactobacillus bulgaricus*, a shift towards lactate formation occurred with a decrease in pH of the growth medium (21). A different regulatory pattern was noticed in *A. succiniciproducens*, with a shift towards lactate and ethanol production associated with neutral pH values and limited CO_2 levels.

Under limited CO_2-HCO_3^- conditions, *A. succiniciproducens* forms lactate and ethanol as electron sink products. Under conditions of excess carbon dioxide, succinate biosynthesis followed a rate pattern (Fig. 2) which was growth rate linked and was 24 times higher than the non-growth-rate-associated formation rate coefficient, which links energy metabolism to nongrowth activities and depends on cell density alone. Under carbon dioxide-limited conditions, lactate was produced at a maximal specific growth rate which was about three times lower than the maximal specific growth rate at which succinate was produced under excess CO_2-HCO_3^- conditions. The growth-related lactate formation rate coefficient (K = 1 g/g of cells per h) is much lower than that of succinate. The growth rate-linked and non-growth-rate-associated lactate production rate coefficients were equal, indicating that similar portions of the energy produced are diverted for growth and for nongrowth purposes and that the electron sink metabolism is maximal under CO_2-HCO_3^- limitation.

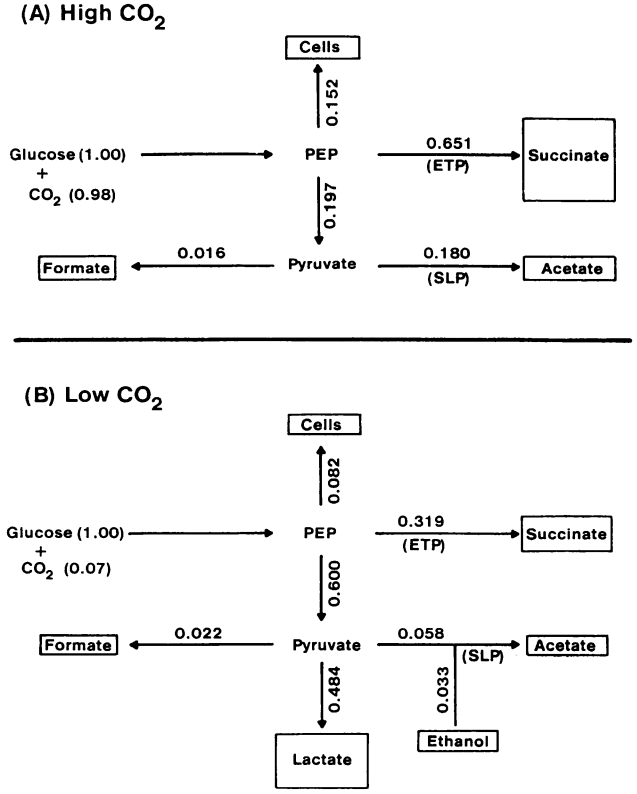

FIG. 4. Comparison of high versus low CO_2-HCO_3^- levels on carbon flux during glucose fermentation by *A. succiniciproducens*. Numbers represent the fraction of glucose carbon participating in various metabolic routes from 1 mol of glucose utilized. Values were calculated from results shown in Table 1.

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