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Dehydrogenases Involved in the Conversion of Succinate to 4-Hydroxybutanoate by *Clostridium kluyveri*

RANDAL A. WOLFF,[1] GERALD W. URBEN,[1]† SEAN M. O'HERRIN,[2] AND WILLIAM R. KENEALY[1,2]*

Department of Biochemistry[1] and Department of Bacteriology,[2] University of Wisconsin, 420 Henry Mall, Madison, Wisconsin 53706

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A pathway of succinate fermentation to acetate and butanoate (butyrate) in *Clostridium kluyveri* has been supported by the results of ^{13}C nuclear magnetic resonance studies of the metabolic end products of growth and the detection of dehydrogenase activities involved in the conversion of succinate to 4-hydroxybutanoate (succinic semialdehyde dehydrogenase and 4-hydroxybutanoate dehydrogenase). *C. kluyveri* fermented [1,4-^{13}C]succinate primarily to [1-^{13}C]acetate, [2-^{13}C]acetate, and [1,4-^{13}C]butanoate. Any pathway proposed for this metabolism must account for the reduction of a carboxyl group to a methyl group. Succinic semialdehyde dehydrogenase activity was demonstrated after separation of the crude extracts of cells grown on succinate and ethanol (succinate cells) by anaerobic nondenaturing polyacrylamide gel electrophoresis. 4-Hydroxybutanoate dehydrogenase activity in crude extracts of succinate cells was detected and characterized. Neither activity was found in cells grown on acetate and ethanol (acetate cells). Analysis of cell extracts from acetate cells and succinate cells by sodium dodecyl sulfate-polyacrylamide gel electrophoreses showed that several proteins were present in succinate cell extracts that were not present in acetate cell extracts. In addition to these changes in protein composition, less ethanol dehydrogenase and hydrogenase activity was present in the crude extracts from succinate cells than in the crude extracts from acetate cells. These data support the hypothesis that *C. kluyveri* uses succinate as an electron acceptor for the reducing equivalents generated from the ATP-producing oxidation of ethanol.

Clostridium kluyveri was originally isolated from an enrichment culture containing ethanol and 20% (wt/vol) yeast extract as the carbon sources. The major products of growth on yeast extract and ethanol were butanoate (butyrate) and hexanoate (caproate), but some butanol and hexanol were also produced. *C. kluyveri* metabolized nonvolatile acids from the yeast extract, but these acids were not further identified (1). Subsequent characterization of the nutrient requirements of this isolate indicated that the need for yeast extract in the growth medium could be eliminated by the addition of acetate, biotin, and *p*-aminobenzoic acid. On this defined medium, *C. kluyveri* produced only trace amounts of butanol and hexanol (16).

Kenealy and Waselefsky found that *C. kluyveri* ferments succinate and ethanol to acetate, butanoate, hexanoate, and smaller amounts of hexanol and butanol and suggested that succinate was one of the nonvolatile acids metabolized from the yeast extract in the original isolation (5). Their work also showed that an increase in cell mass did not occur upon an increase in succinate consumption, the latter having been stimulated by the provision of reducing equivalents in the form of hydrogen gas. This finding indicated that succinate degradation does not directly yield any net ATP but can be used as an electron sink to oxidize electron carriers (5).

Kenealy and Waselefsky proposed a new reductive pathway of succinate metabolism by *C. kluyveri* on the basis of the results of their growth studies. In this proposed pathway, 2 reducing equivalents and 1 ATP equivalent are consumed when a carboxyl group of succinate is reduced to the hydroxyl group of 4-hydroxybutanoate. The further metabolism of 4-hydroxybutanoate to acetate or butanoate has been described for *C. kluyveri* (2, 14). When acetate is produced from 4-hydroxybutanoate, 1 reducing equivalent and 1 ATP equivalent are generated. Therefore, if one molecule of succinate were metabolized to two molecules of acetate by the proposed pathway, 1 reducing equivalent would be consumed, with no net change in ATP equivalents. The fermentation of two succinate molecules to four acetate molecules with the concurrent consumption of 2 reducing equivalents would allow the generation of one ATP by the oxidation of one molecule of ethanol to one molecule of acetate.

Succinate is a common product of anaerobic fermentations, but only a few anaerobic organisms are known to metabolize succinate to provide energy for growth. All of the known pathways for catabolic metabolism of succinate involve a decarboxylation. *Propionigenium modestum* and *Sporomusa malonica* convert succinate to propionate (3, 11). In *P. modestum*, the decarboxylation of methylmalonyl-coenzyme A (CoA) derived from succinate has been shown to be coupled to sodium ion extrusion and sodium ion-dependent ATP synthesis (4). Several *Desulfovibrio* strains that reduce sulfur compounds have been shown to oxidize succinate to malate and metabolize malate to pyruvate via a malic enzyme that uses decarboxylation (7).

We report here ^{13}C proton-decoupled nuclear magnetic resonance (NMR) experiments wherein the metabolism of [1,4-^{13}C]succinate by *C. kluyveri* is outlined. We also describe a 4-hydroxybutanoate dehydrogenase activity in crude cell extracts and a succinic semialdehyde dehydrogenase activity in preparations from native gel electrophoresis of crude cell extracts. These activities and the production of [1-^{13}C]acetate and [2-^{13}C]acetate from [1,4-^{13}C]succinate support the pathway proposed by Kenealy and Waselefsky

* Corresponding author.
† Present address: Kraft General Foods, Glenview, IL 60025.

(5). To explain the production of [1,4-^{13}C]butanoate from [1,4-^{13}C]succinate, a variation of the pathway is proposed. A preliminary report of these results has already been presented elsewhere (18).

MATERIALS AND METHODS

Media and growth conditions. *C. kluyveri* ATCC 8527 was routinely cultivated in CM-5 medium (17) with the mineral elixir described by Kenealy and Zeikus (6), 0.05% (wt/vol) Difco yeast extract, and 0.002 g of resazurin per liter. The medium was dispensed under a gas phase of N_2-CO_2 (95/5). After sterilization, the medium was reduced with 0.025% (wt/vol) cysteine. Ethanol (200 mM) and a cosubstrate (50 mM acetate or 25 mM succinate) were then added. For large-scale culture, 12-liter carboys of CM-5 were used. After sterilization, the carboys were sparged with N_2-CO_2 (95/5). The limiting substrate (ethanol [60 mM]), cysteine (0.025% [wt/vol]), and the cosubstrate (succinate or acetate [150 mM]) were added by injection of sterilized anaerobic solutions. The pH was kept between 6.3 and 6.8, and the temperature was kept between 28 and 30°C. Cells were harvested by centrifugation at late-log phase, frozen in liquid nitrogen, and placed under N_2 in anaerobic tubes (Bellco Glass, Vineland, N.J.), and the tubes were stored at −70°C.

Analysis of fermentation substrates and products. Volatile acids and alcohols were analyzed by gas-liquid chromatography. Samples (200 μl) in sealed vials were acidified with 300 μl of 1 M H_3PO_4, and 3 μl was then injected into a Hewlett-Packard 5890 series II gas chromatograph equipped with a 7673A automatic liquid sampler. The following chromatographic conditions were used: stainless steel column (inside diameter, 1.8 m by 0.32 cm) packed with GP 10% SP-1200–1% H_3PO_4 on 80/100 Chromosorb W AW (Supelco, Bellefonte, Pa.); helium carrier gas at 20 ml min^{-1}; injector temperature and flame ionization detector temperature, 200°C. The oven temperature program followed the method described by Weimer (17). Retention times were 1.95 min for ethanol, 7.42 min for acetate, 9.7 min for butanoate, and 15.5 min for hexanoate. 4-Hydroxybutanoate was converted to its methyl ester and analyzed by gas chromatography by the method described by Kunz and Weimer (8). A stainless steel column (inside diameter, 1.8 m by 0.32 cm) packed with 10% SP-2340 on 100/120 Chromosorb W AW (Supelco) and isothermal gradient (160°C) were used. The Hewlett-Packard automatic sampler injected 2-μl samples from the chloroform layer. The retention time for 4-hydroxybutanoate was 4.3 min. Succinate was analyzed by a Bio-Rad HPX-87H column at 45°C with 28 mM H_2SO_4 as the eluant, a flow rate of 0.6 ml min^{-1}, a 20-μl injection volume, and a UV monitor at 210 nm.

^{13}C NMR. *C. kluyveri* was grown in medium containing 0.025% (wt/vol) cysteine, 20 mM [1,4-^{13}C]succinate (99.7 atom% ^{13}C; Isotec Inc., Miamisburg, Ohio), and 20 or 200 mM ethanol. Media were inoculated (1% [vol/vol]) with the same growing stock culture. Samples of the medium were filter sterilized at various times and stored at −20°C for further analysis. Proton-decoupled (wide-band alternating-phase low-power technique for zero residue splitting) one-dimensional ^{13}C NMR spectra were obtained on a Bruker AM-500 NMR spectrometer operating at 125.759 MHz and with a deuterium lock. Measurements were made at 25°C on pH 7 samples containing 10% deuterium oxide. Acquisition conditions were as follows: 45° pulses, 25-kHz sweep width, 8,192 time domain points (0.164-s acquisition time), 2.5-s relaxation delay, and 256 scans. Free-induction decays were apodized by exponential multiplication (line broadening, 10 Hz) and were Fourier transformed. Chemical shifts were set with respect to an external standard of [2-^{13}C]acetate at 23.596 ppm.

Cell extracts. Frozen cells were resuspended in anaerobic distilled water and broken with a French press at 200,000 kPa. Anaerobic conditions as discussed by Zeikus et al. (20) were used throughout handling of the cell extracts. Vessels and syringes were rinsed with 1 mM sodium hydrosulfite before they were used to handle cell extracts. Extracts were centrifuged at 5,000 × *g* for 15 min at 4°C to remove whole cells. The pellet from a second centrifugation at 18,500 × *g* for 60 min was resuspended in the same volume of distilled water as the supernatant volume. The supernatant and resuspended pellet were stored separately at −20°C. The protein compositions of the supernatant and pellet fractions were analyzed by sodium dodecyl sulfate-12% polyacrylamide gel electrophoreses (SDS-PAGE) and silver stained by the method described by Wray et al. (19).

Enzyme assays. All assays were performed with 1-ml volumes at 30°C and initiated with substrate after a preincubation at 30°C, with the exception of the hydrogenase assay, which was initiated with cell extract. Oxidation or reduction of NAD or NADP (ε = 6,200 M^{-1} cm^{-1}) was measured at 340 nm. Reduction of methyl viologen (ε = 9,200 M^{-1} cm^{-1}) was measured at 578 nm. Protein concentrations were determined by the method described by Lowry et al. (9), standardized with bovine serum albumin.

The end products of 1-ml reactions were analyzed to confirm 4-hydroxybutanoate dehydrogenase activity. The reaction mixture consisted of 50 mM PIPES [piperazine-1,1′-bis(2-ethanesulfonic acid)] buffer (pH 6.5)–20 mM succinic semialdehyde–2 mM NADH–2 mM dithiothreitol–supernatant from cell extract of succinate cells. The reaction was stopped with 5% (wt/vol) trichloroacetic acid after 5 min, and the supernatant was analyzed.

Native gel electrophoresis was performed by the method described by Ritchie et al. (10), except for the following changes. The Tris buffer concentrations were 0.2 M, pH 8.8, in the 10% acrylamide running gel and 0.125 M, pH 6.8, in the stacking gel. The ratio of acrylamide to *N,N′*-methylenebisacrylamide was 40:1. Samples were loaded anaerobically in 20% (vol/vol) glycerol–700 mM β-mercaptoethanol–0.15 M Tris (pH 6.8). The running buffer did not contain any detergent but did contain 1 mM sodium hydrosulfite. The gel was run for 5 h at 40 mV under a nitrogen atmosphere, removed from the gel holder, and sliced along lanes. Lanes to be tested for activity were immersed in 5 ml of 100 mM anaerobic CAPS (cyclohexylaminopropane sulfonic acid) buffer (pH 10)–10 mM $NADP^+$–0.5 mM CoA–20 mM succinic semialdehyde. Control lanes were immersed in reaction mix without either succinic semialdehyde or CoA. After incubation, the gel slices were irradiated with long-wave UV light to visualize fluorescent NADPH bands, the bands were marked, and the remaining gel was cut at the location corresponding to the fluorescent band. These gel slices were minced by passing them through a syringe and were extracted with an equal volume of water at 0°C for 16 h. The gel slice extract was drawn off and frozen at −20°C.

The activity of succinic semialdehyde dehydrogenase in the $NADP^+$-reducing direction was measured with 50 mM CAPS buffer (pH 10)–0.4 mM dithiothreitol–0.5 mM $NADP^+$–0.25 mM CoA–10 mM succinic semialdehyde. The activity of succinic semialdehyde dehydrogenase in the NADPH-oxidizing direction was measured with 50 mM

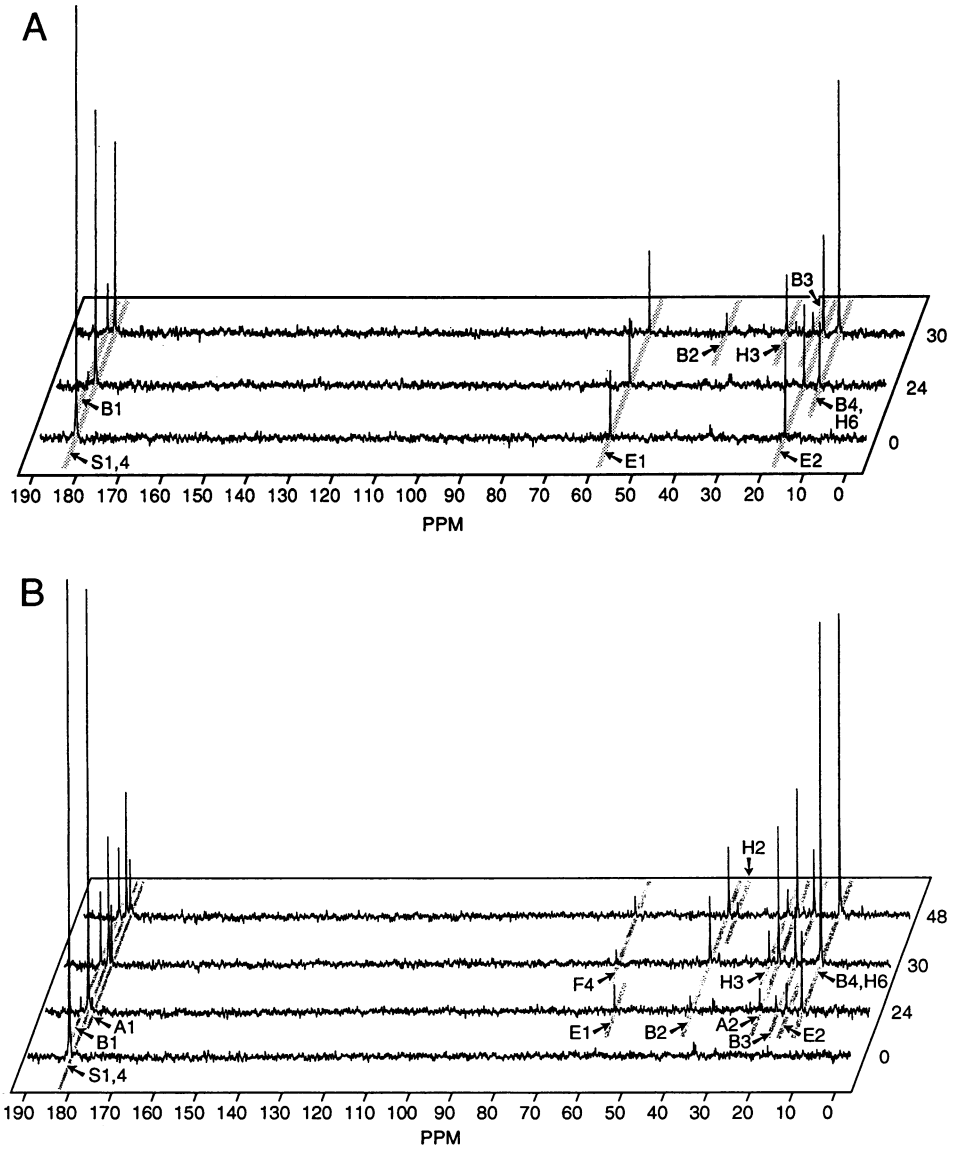

FIG. 1. ^{13}C NMR spectra of medium samples from growth of *C. kluyveri* on [1,4-^{13}C]succinate and ethanol. (A) High ethanol; (B) low ethanol. A, acetate; B, butanoate; E, ethanol; F, 4-hydroxybutanoate; H, hexanoate; S, succinate. The numbers following the one-letter abbreviations give the positions of the carbon in the molecule. The resonance of 4-hydroxybutanoate carbon 1, which lies between the butanoate carbon 1 and the succinate carbon 1,4 resonances, is not labeled in panel B. These spectra are representative of the results of replicate experiments.

PIPES buffer (pH 6.9)–2 mM dithiothreitol–0.2 mM NADPH–0.2 mM succinyl-CoA.

For comparison between cell extracts, the activity of 4-hydroxybutanoate dehydrogenase in the NAD^+-reducing direction was measured with 50 mM CAPS buffer (pH 10)–0.4 mM dithiothreitol–0.5 mM NAD^+–10 mM 4-hydroxybutanoate. The activity of 4-hydroxybutanoate dehydrogenase in the NADH-oxidizing direction was measured with 50 mM PIPES buffer (pH 6.5)–0.4 mM dithiothreitol–0.3 mM NADH–10 mM succinic semialdehyde.

Alcohol dehydrogenase was measured with 50 mM Tris buffer (pH 8.2)–0.4 mM dithiothreitol–0.5 mM NAD^+–100 mM ethanol. Hydrogenase was measured under a hydrogen gas phase with 50 mM MOPS [3-(*N*-morpholino)propanesulfonic acid] buffer (pH 7.2)–0.4 mM dithiothreitol–1 mM methyl viologen. Sodium hydrosulfite was added until the solution turned light blue before the hydrogenase assay was initiated.

RESULTS

Products of [1,4-^{13}C]succinate fermentation. When grown on [1,4-^{13}C]succinate and high levels of ethanol, *C. kluyveri* converted [1,4-^{13}C]succinate primarily to [1,4-^{13}C]butanoate. Smaller amounts of [2,3-^{13}C]butanoate and [3,6-^{13}C]hexanoate were also observed (Fig. 1A). When *C. kluyveri* was grown on [1,4-^{13}C]succinate and low levels of ethanol, in addition to the products seen with growth on high levels of ethanol, it converted [1,4-^{13}C]succinate to

[1-^{13}C]acetate, [2-^{13}C]acetate, and smaller amounts of [1,4-^{13}C]4-hydroxybutanoate (Fig. 1B).

To normalize the percentage of ^{13}C at each position in the sample, the NMR spectra of natural abundance-labeled standards were acquired under conditions identical to those used for analyzing the samples. The ratios of the signal intensities of the natural abundance-labeled butanoate carbons 1 through 4 were found to be 1:4.7:4.3:3.8, respectively. Similarly, the ratios of signal intensities of the natural abundance-labeled hexanoate carbons 1 through 6 were found to be 1:4.7:4.9:7.0:7.8:7.1, respectively, and the ratio between the signals from the natural abundance-labeled acetate carbons 1 and 2 was found to be 1:2.6.

The resonances of the butanoate carbon 4 and hexanoate carbon 6 were coincident, but the contributions from each compound to the combined signal could be resolved if it was assumed that the hexanoate observed in the samples was made from the condensation of butanoate and acetate (12). This assumption implies that hexanoate would have the same labeling ratio between its carbon 3 and carbon 6 as butanoate has between its carbons 1 and 4. Therefore, the percentage of label in the hexanoate carbon 6 could be estimated from the percentage of label in the hexanoate carbon 3.

The labeling pattern under high-ethanol conditions (Fig. 1A) showed that the butanoate carbons 2 and 3 contained approximately equal percentages of label. The concentrations of label in the butanoate carbons 1 and 4 also were found to contain approximately equal percentages of label. However, the butanoate carbons 1 and 4 contained approximately four times more label than the carbons 2 and 3 under these conditions of growth. Label in the hexanoate carbons 3 and 6 could be detected after 30 h of growth. No labeled acetate was detected.

Under low-ethanol conditions (Fig. 1B), the labeling pattern seen in butanoate was the same as the pattern seen in butanoate under the high-ethanol condition. In addition, acetate was found to be labeled equally in the carbons 1 and 2. Label was not quantified in the minor peaks 4-hydroxybutanoate carbon 1, 4-hydroxybutanoate carbon 4, or hexanoate carbon 2. No peak was detected for the hexanoate carbon 1. ^{13}C label in the hexanoate carbon 1 equal to that found in the hexanoate carbon 2 would not have been detected by our methods because of the weakness of the NMR signal produced by carboxyl carbons.

Acetate was the major end product when *C. kluyveri* was grown on succinate and low amounts of ethanol, whereas butanoate was the major end product when *C. kluyveri* was grown on succinate and high amounts of ethanol. However, acetate did reach significant concentrations even under the high-ethanol condition (Fig. 2). The acetate in the medium under the high-ethanol condition must have been derived primarily from carbon sources other than succinate (presumably ethanol), because even when it was present at concentrations similar to those found in the low-ethanol condition, it was still not detected by ^{13}C NMR. The production of butanoate exceeds the consumption of succinate under the high-ethanol condition, which implies that some of the carbons of butanoate must also have been derived from a carbon source other than succinate (presumably ethanol and acetate formed from ethanol).

C. kluyveri continuously consumed ethanol under high- and low-ethanol conditions. It consumed ethanol at a much faster rate than succinate under high-ethanol conditions but at a rate similar to that for succinate under low-ethanol conditions (Fig. 2). However, Fig. 1B shows that *C. kluyveri*

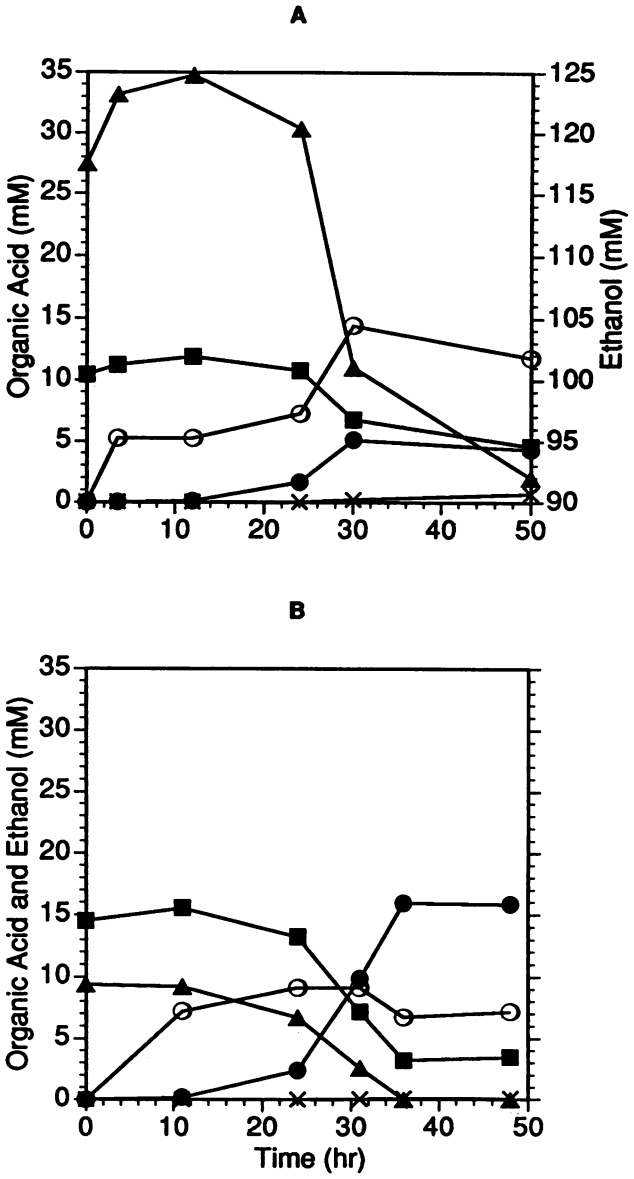

FIG. 2. Analysis of medium samples from growth of *C. kluyveri* on [1,4-^{13}C]succinate and ethanol. (A) High ethanol; (B) low ethanol. Symbols: ●, acetate; ○, butanoate; ▲, ethanol; X, hexanoate; ■, succinate.

produced labeled ethanol between 0 and 24 h under the low-ethanol condition.

Characterization of activities. Extracts from succinate cells were screened for the presence of oxidoreductases predicted by the pathway proposed by Kenealy and Waselefsky (5). Production of NADH was seen when NAD^+ and 4-hydroxybutanoate were added to crude extracts. The discovery of 4-hydroxybutanoate dehydrogenase activity was confirmed by an oxidation of NADH upon the addition of succinic semialdehyde by crude extracts. Approximately one-third of the activity was seen when NADP(H) was substituted for NAD(H). Gas-chromatographic analysis of the end products of the conversion of succinic semialdehyde to 4-hydroxybutanoate dependent on NADH showed that 1 mM 4-hydroxybutanoate was produced. Exposure of the crude extracts to

TABLE 1. Comparison of 4-hydroxybutanoate dehydrogenase, hydrogenase, and ethanol dehydrogenase activities in cell extracts from acetate cells and succinate cells[a]

Cell extract source and fraction	Ethanol dehydrogenase	Hydrogenase	4-Hydroxybutanoate dehydrogenase ($NAD^+ \rightarrow NADH$)	4-Hydroxybutanoate dehydrogenase ($NADH \rightarrow NAD^+$)
Acetate pellet	70 ± 30	127 ± 9	—[b]	—[b]
Acetate supernatant	160 ± 90	370 ± 70	—[b]	4 ± 4
Succinate pellet	10 ± 10	20 ± 10	1 ± 1	160 ± 50
Succinate supernatant	4 ± 5	28 ± 2	70 ± 40	350 ± 80

[a] All values are in nanomoles milligram^{-1} minute^{-1} and are reported as the averages ± the standard deviations from eight repetitions.
[b] —, below limits of detection (1 nmol mg^{-1} min^{-1}).

air destroyed the 4-hydroxybutanoate dehydrogenase activity. Under the conditions of the assay, activity versus protein concentration was linear.

NADPH oxidation was detected when succinyl-CoA was added to crude extracts of succinate cells (105 ± 5 nmol mg^{-1} min^{-1}), but $NADP^+$ reduction could not be detected when succinic semialdehyde and CoA were added to crude extracts of succinate cells. Succinic semialdehyde dehydrogenase activity was observed after separation of proteins in the crude extract by native gel electrophoresis. Gel segments immersed in buffer, succinic semialdehyde, CoA, and $NADP^+$ exhibited a diffuse fluorescent NADPH band dependent on the addition of all three substrates. Addition of 4-hydroxybutanoate and NAD revealed a sharp fluorescent band also dependent on both substrates but separate from the succinic semialdehyde activity. Neither succinic semialdehyde dehydrogenase activity nor 4-hydroxybutanoate dehydrogenase activity was observed when extracts of acetate cells were separated (on the same gel as succinate cells). Lanes containing either acetate or succinate extracts contained a band that was able to reduce methyl viologen when hydrogen was added.

Extracts of these gel slices contained 90 ± 30 nmol of succinic semialdehyde dehydrogenase activity mg^{-1} min^{-1} in the $NADP^+$-reducing direction and 45 ± 15 nmol of activity mg^{-1} min^{-1} in the NADPH-oxidizing direction. No succinic semialdehyde dehydrogenase activity was seen with NAD(H) and extracts from gel slices. Exposure of the extracts to air destroyed succinic semialdehyde dehydrogenase activity.

Hydrogenase, ethanol dehydrogenase, and 4-hydroxybutanoate dehydrogenase were measured in crude extracts from acetate cells and succinate cells. Hydrogenase activity and ethanol dehydrogenase activity were lower in extracts of succinate cells than in extracts of acetate cells (Table 1). Most of the 4-hydroxybutanoate dehydrogenase activity was found in the supernatant fraction of cell extracts from succinate cells. Only a small amount of NADH oxidation was detected when 4-hydroxybutanoate dehydrogenase was assayed with extracts from acetate cells. When crude extracts from cells that had been grown on acetate and ethanol for 48 h after growth on succinate and ethanol were assayed, the 4-hydroxybutanoate dehydrogenase activity was found to have declined to undetectable levels from the high levels seen with growth on succinate and ethanol.

These extracts were also analyzed by SDS-PAGE. As can be seen in Fig. 3, the protein banding patterns of the extract supernatant from acetate cells before and after succinate growth were similar. Several additional bands were present only under the succinate growth condition. Proteins with approximate molecular masses of more than 100, 66, 58, 55, 37, and 30 kDa were indicated. The more than 100-kDa band was more distinct in a similar comparison between pellet fractions (data not shown).

DISCUSSION

C. kluyveri converted succinate to butanoate as well as acetate during growth on succinate and ethanol. The ^{13}C NMR data show that the succinate carbons 1 and 4 are metabolized to the butanoate carbons 1 and 4 as well as the acetate carbons 1 and 2. In both cases, one of the carboxyl carbons of succinate is reduced to a methyl carbon, which is not consistent with succinate degradation by the complete tricarboxylic acid cycle or the glyoxylate cycle. Appearance of label in the acetate carbons 1 and 2 under low-ethanol conditions is consistent with the metabolism proposed by Kenealy and Waselefsky (5), as is the appearance of label in the minor product 4-hydroxybutanoate at the carbon positions 1 and 4. The appearance of [1,4-^{13}C]butanoate and the

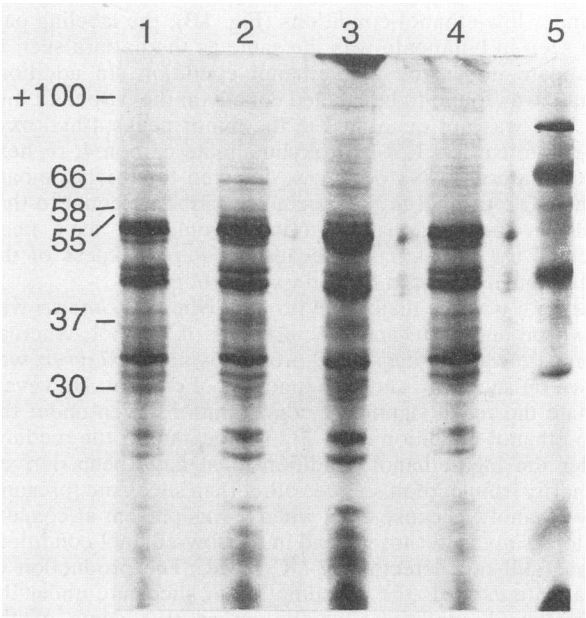

FIG. 3. Cell extracts from acetate cells and succinate cells were analyzed by SDS-PAGE and silver staining. All cell extracts are from high-ethanol conditions. Lanes: 1, acetate cells; 2, cells grown for 2 days on succinate with an inoculum from the culture used in lane 1; 3, cells grown for 2 days on succinate with an inoculum from the culture used in lane 2; 4, cells grown for 2 days on acetate also with an inoculum from the culture used in lane 2; 5, Bio-Rad low-molecular-weight standards. Molecular weights correspond to bands seen only in extracts from succinate cells.

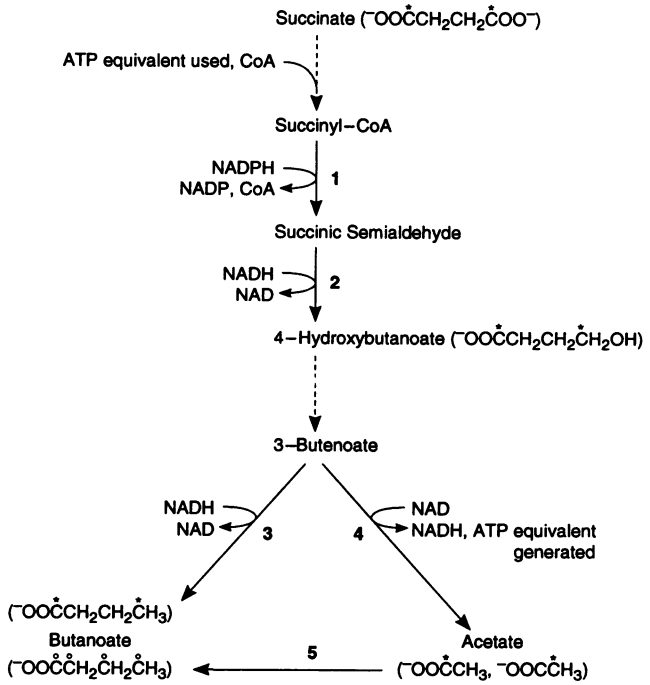

FIG. 4. Hypothetical pathway consistent with ^{13}C NMR data and enzymatic studies. Dashed arrows indicate activities not yet observed in *C. kluyveri* cell extracts. Asterisks and open circles mark the positions of label observed in these compounds during ^{13}C NMR experiments. Randomization would result in the positions marked by open circles having one-half the label of positions marked by asterisks. 1, succinic semialdehyde dehydrogenase; 2, 4-hydroxybutanoate dehydrogenase; 3 and 4, the conversion of 3-butenoate (vinyl acetate) to butanoate or acetate, respectively, in *C. kluyveri* (described by Bartsch and Barker [2] and Stadtman and Barker [14]); 5, the incorporation of acetate into butanoate (described by Thauer et al. [15] and Smith et al. [12]).

absence of labeled acetate under high-ethanol conditions indicate a variation on the pathway proposed by Kenealy and Waselefsky.

The labeling pattern found in butanoate limits the possible routes of its production from succinate. If butanoate were formed from acetate and ethanol by known routes of acetate and ethanol metabolism, equal amounts of label would be expected in each of the butanoate carbons (12), provided that [1-^{13}C]acetate and [2-^{13}C]acetate derived from [1,4-^{13}C]succinate inside the cell are in the same pool as acetate outside the cell (Fig. 4). While the occurrence of label in all of the carbons of butanoate indicates that this metabolism is occurring, the majority of label is in carbons 1 and 4, which indicates a conversion of succinate into butanoate without a step that breaks the four-carbon chain. The conversion of succinate to 4-hydroxybutanoate followed by the conversion of 4-hydroxybutanoate to butanoate as previously described by Bartsch and Barker (2) and Stadtman and Barker (14) would result in the butanoate carbons 1 and 4 being labeled (Fig. 4).

In the low-ethanol condition, *C. kluyveri* produced labeled ethanol between certain time points while it continued to consume ethanol, as shown by gas-chromatographic analysis. Under these conditions, acetyl-CoA derived from [1,4-^{13}C]succinate is present, and thus, ethanol derived from this labeled acetyl-CoA could also be labeled. This finding is consistent with the results described by Smith et al. (12), who hypothesized that production of labeled ethanol from labeled acetate in *C. kluyveri* is a process of isotopic equilibration through reversible reactions rather than a net synthesis of ethanol from acetate.

New proteins were induced by growth on succinate, and two enzymatic activities (4-hydroxybutanoate dehydrogenase and succinic semialdehyde dehydrogenase) were found in succinate cell extracts and not in acetate cell extracts. Evidence for a 4-hydroxybutanoate dehydrogenase activity consists of the following: (i) NAD^+ reduction was dependent on 4-hydroxybutanoate, (ii) NADH oxidation was dependent on succinic semialdehyde, and (iii) 4-hydroxybutanoate was formed when extracts were exposed to succinic semialdehyde and NADH. Evidence for a succinic semialdehyde dehydrogenase activity consists of the following: (i) $NADP^+$ reduction was dependent on succinic semialdehyde and CoA, and (ii) NADPH oxidation was dependent on succinyl-CoA. The presence of this enzyme has been independently confirmed by Söhling and Gottschalk, who have purified it to homogeneity (13).

NADPH oxidation dependent on succinyl-CoA addition was detected in crude extracts of succinate cells; however, the oxidation of NADPH could not be completely attributed to succinic semialdehyde dehydrogenase activity, since the product of succinic semialdehyde dehydrogenase activity (succinic semialdehyde) is known to cause the oxidation of NADPH when it is added to crude extracts. Similarly, activities present in the crude extract also may have prevented the detection of succinic semialdehyde dehydrogenase activity in the $NADP^+$-reducing direction. For example, when succinic semialdehyde, CoA, and $NADP^+$ were added to crude extracts, for every $NADP^+$ reduced to NADPH by the conversion of succinic semialdehyde to succinyl-CoA, a NADPH molecule could be oxidized to $NADP^+$ through the action of 4-hydroxybutanoate dehydrogenase and enzymes known to interconvert NADPH and NADH.

The difference in 4-hydroxybutanoate dehydrogenase activity between the supernatant and the pellet fractions is greater when it is measured in the NAD^+-reducing direction than in the NADH-oxidizing direction (Table 1). This difference indicates a change in 4-hydroxybutanoate dehydrogenase activity caused by the fractionation or the presence of either another NADH-oxidizing activity in the pellet or another NAD^+-reducing activity in the supernatant. Further studies of purified enzymes will allow better characterization of the discrete enzymes that participate in the conversion of succinate to 4-hydroxybutanoate.

Three activities were measured for comparison between crude extracts of succinate cells and acetate cells. 4-Hydroxybutanoate dehydrogenase activity, which is present in the extracts of succinate cells, is not necessary for the growth of *C. kluyveri* on acetate and ethanol. The reduction in hydrogenase activity under conditions of succinate and ethanol growth substantiates the proposed purpose of the succinate pathway: to use succinate instead of protons as an electron acceptor. Ethanol dehydrogenase activity is necessary for ATP production both in acetate and ethanol and in succinate and ethanol metabolism (5). However, the reduction in ethanol dehydrogenase activity under conditions of succinate and ethanol growth may be due to a reduced need for the production of butanoate from acetate and ethanol, a free-energy-producing pathway (15), because the oxidation of ethanol with the reduction of succinate provides more free

energy than the oxidation of ethanol with the reduction of protons.

C. kluyveri metabolized succinate to butanoate under high-ethanol conditions and to acetate under low-ethanol conditions. Figure 4 shows a hypothetical pathway which *C. kluyveri* may be using for succinate metabolism. In this pathway, when two molecules of succinate are converted to four molecules of acetate, 2 reducing equivalents are consumed, which allows one molecule of ethanol to be oxidized to one molecule of acetate, with the net production of one ATP equivalent. When two molecules of succinate are converted to two molecules of butanoate, 6 reducing equivalents are consumed, which allows three molecules of ethanol to be oxidized to three molecules of acetate, producing 3 ATP equivalents. Since the conversion of two molecules of succinate to two molecules of butanoate consumes 2 ATP equivalents, the net yield for this metabolism is 1 ATP equivalent. Therefore, the theoretical ATP yield for succinate use is the same whether acetate or butanoate is produced.

The key to why *C. kluyveri* would use one pathway instead of another is to understand the regulation of the enzymes in those pathways. The levels of hydrogenase, ethanol dehydrogenase, succinic semialdehyde dehydrogenase, and 4-hydroxybutanoate dehydrogenase activity as well as the appearance of several new proteins under succinate growth are all examples of regulation in this anaerobe that deserve further study.

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REFERENCES

1. **Barker, H. A., and S. M. Taha.** 1941. *Clostridium kluyveri*, an organism concerned in the formation of caproic acid from ethyl alcohol. J. Bacteriol. **43:**347–363.
2. **Bartsch, R. G., and H. A. Barker.** 1961. A vinylacetyl isomerase from *Clostridium kluyveri*. Arch. Biochem. Biophys. **92:**122–132.
3. **Dehning, I., M. Stieb, and B. Schink.** 1989. *Sporomusa malonica* sp. nov., a homoacetogenic bacterium growing by decarboxylation of malonate or succinate. Arch. Microbiol. **151:**421–426.
4. **Hilpert, W., B. Schink, and P. Dimroth.** 1984. Life by a new decarboxylation-dependent energy conservation mechanism with Na^+ as coupling ion. EMBO J. **3:**1665–1670.
5. **Kenealy, W. R., and D. M. Waselefsky.** 1985. Studies on the substrate range of *Clostridium kluyveri*; the use of propanol and succinate. Arch. Microbiol. **141:**187–194.
6. **Kenealy, W. R., and J. G. Zeikus.** 1981. Influence of corrinoid antagonists on methanogen metabolism. J. Bacteriol. **146:**133–140.
7. **Kremer, D. R., H. E. Nienhuis-Kuiper, C. J. Timmer, and T. A. Hansen.** 1989. Catabolism of malate and related dicarboxylic acids in various *Desulfovibrio* strains and the involvement of an oxygen-labile NADPH dehydrogenase. Arch. Microbiol. **151:**34–39.
8. **Kunz, D. A., and P. J. Weimer.** 1983. Bacterial formation and metabolism of 6-hydroxyhexanoate evidence of a potential role for ω-oxidation. J. Bacteriol. **156:**567–575.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
10. **Ritchie, R. F., J. G. Harter, and T. B. Bayles.** 1966. Refinements of acrylamide electrophoresis. J. Lab. Clin. Med. **68:**842–850.
11. **Schink, B., and N. Pfennig.** 1982. *Propionigenium modestum* gen. nov. sp. nov. a new strictly anaerobic nonsporing bacterium growing on succinate. Arch. Microbiol. **133:**209–216.
12. **Smith, G. M., B. W. Kim, A. A. Franke, and J. D. Roberts.** 1985. ^{13}C NMR studies of butyric fermentation in *Clostridium kluyveri*. J. Biol. Chem. **260:**13509–13512.
13. **Söhling, B., and G. Gottschalk.** 1993. Purification and characterization of a coenzyme-A-dependent succinate-semialdehyde dehydrogenase from *Clostridium kluyveri*. Eur. J. Biochem. **212:**121–127.
14. **Stadtman, E. R., and H. A. Barker.** 1949. Fatty acid synthesis by enzyme preparations of *Clostridium kluyveri*. J. Biol. Chem. **181:**221–235.
15. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100–180.
16. **Thauer, R. K., K. Jungermann, H. Henninger, J. Wenning, and K. Decker.** 1968. The energy metabolism of *Clostridium kluyveri*. Eur. J. Biochem. **4:**173–180.
17. **Weimer, P. J.** 1984. Control of product formation during glucose fermentation by *Bacillus macerans*. J. Gen. Microbiol. **130:**103–111.
18. **Wolff, R. A., G. Urben, S. M. O'Herrin, and W. R. Kenealy.** 1992. A new anaerobic pathway for succinate degradation in *Clostridium kluyveri*, abstr. O-32, p. 314. Abstr. 92nd Annu. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
19. **Wray, W., T. Boulikas, V. P. Wray, and R. Hancock.** 1981. Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118:**197–203.
20. **Zeikus, J. G., G. Fuchs, W. Kenealy, and R. K. Thauer.** 1977. Oxidoreductases involved in cell carbon synthesis of *Methanobacterium thermoautotrophicum*. J. Bacteriol. **132:**604–613.