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Pathway of Propionate Oxidation by a Syntrophic Culture of *Smithella propionica* and *Methanospirillum hungatei*

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The pathway of propionate conversion in a syntrophic coculture of *Smithella propionica* and *Methanospirillum hungatei* JF1 was investigated by ^{13}C-NMR spectroscopy. Cocultures produced acetate and butyrate from propionate. [3-^{13}C]propionate was converted to [2-^{13}C]acetate, with no [1-^{13}C]acetate formed. Butyrate from [3-^{13}C]propionate was labeled at the C2 and C4 positions in a ratio of about 1:1.5. Double-labeled propionate (2,3-^{13}C) yielded not only double-labeled acetate but also single-labeled acetate at the C1 or C2 position. Most butyrate formed from [2,3-^{13}C]propionate was also double labeled in either the C1 and C2 atoms or the C3 and C4 atoms in a ratio of about 1:1.5. Smaller amounts of single-labeled butyrate and other combinations were also produced. 1-^{13}C-labeled propionate yielded both [1-^{13}C]acetate and [2-^{13}C]acetate. When ^{13}C-labeled bicarbonate was present, label was not incorporated into acetate, propionate, or butyrate. In each of the incubations described above, ^{13}C was never recovered in bicarbonate or methane. These results indicate that *S. propionica* does not degrade propionate via the methyl-malonyl-coenzyme A (CoA) pathway or any other of the known pathways, such as the acryloyl-CoA pathway or the reductive carboxylation pathway. Our results strongly suggest that propionate is dismutated to acetate and butyrate via a six-carbon intermediate.

In methanogenic environments propionate is oxidized by acetogenic bacteria to acetate and carbon dioxide (16, 18). Methanogenic archaea make this reaction energetically favorable by removing reducing equivalents either as hydrogen or as formate (1, 3, 19). Syntrophic propionate oxidation mainly occurs via the randomizing methyl-malonyl-coenzyme A (CoA) pathway, as was demonstrated for several *Syntrophobacter* species (6, 7, 11), as well as for mixed methanogenic cultures (2, 5, 8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (9, 22, 23). In these studies, evidence was provided that part of the propionate is carboxylated to butyrate which is then degraded to acetate. Alternative possible pathways of propionate conversion were recently documented by Textor et al. (21).

Recently, a novel syntrophic propionate-oxidizing bacterium was isolated which may possess a propionate-degradation pathway via butyrate (10). Cocultures of *Smithella propionica* and a hydrogen- and formate-utilizing methanogen produce less methane and more acetate than cocultures with *Syntrophobacter* strains. In addition, the cocultures with *S. propionica* produce small amounts of butyrate. It was suggested that this organism dismutates propionate to acetate and butyrate followed by syntrophic β-oxidation of butyrate to acetate. We report here the results of ^{13}C-nuclear magnetic resonance (NMR) studies to elucidate the pathway of propionate oxidation in *S. propionica*.

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MATERIALS AND METHODS

Organisms and cultivation. *Methanospirillum hungatei* JF1^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen in Braunschweig, Germany. The MS medium (3) with 0.5 g of casein tryptic peptone and 0.5 g of yeast extract per liter, but with 1 mM L-cysteine instead of 1 mM mercaptoethane sulfonate, was used to grow syntrophic cultures of *S. propionica* and *M. hungatei*. The methanogens were pregrown on H_2 and CO_2 in 120-ml serum vials with 50 ml of medium. After growth the gas atmosphere was replaced by N_2 and CO_2 (80:20), and *S. propionica* (in coculture with *Methanospirillum hungatei*) was inoculated into these *M. hungatei* cultures. The cocultures were incubated at 37°C with 10 mM propionate.

NMR spectroscopy. Stable isotopes (minimum, 99% ^{13}C) were obtained from Campro Scientific B.V. (Veenendaal, The Netherlands). Serum vials were prepared with 10 mM concentrations of either [1-^{13}C]propionate, [2-^{13}C]propionate, [3-^{13}C]propionate, or [2,3-^{13}C]propionate as substrates in 50 ml of medium. To test the incorporation of $H^{13}CO_3^{1-}$, the coculture was grown on 10 mM unlabeled propionate in the presence of 50 mM $H^{13}CO_3^{1-}$. The combination of 10 mM unlabeled propionate and 4 mM [1-^{13}C]acetate or [2-^{13}C]acetate was also tested. After 10, 20, and 30 days 3-ml samples were withdrawn for analysis. Cells were removed by centrifugation at 10,000 × *g*, and D_2O and dioxane were added to 2 ml of supernatant to give a final volume of 2.5 ml in 10-mm (outer-diameter) NMR tubes containing 10% D_2O and 100 mM dioxane. The proton-decoupled ^{13}C-NMR spectra of the samples were recorded at 75.47 MHz on a Bruker AMX-300 NMR spectrometer. For each spectrum 7,200 transients (2 h) were accumulated and stored on disk using 32,000 datum points, a 45° pulse angle (pulse duration, 9 μs), and a delay time of 1 s between the pulses. The measuring temperature was maintained at 25°C, and the chemical shift belonging to the dioxane carbon nuclei (67.4 ppm) was used as an internal standard. The deuterium in the samples (10% [vol/vol]) was used for the field lock. A balance of ^{13}C-labeled compounds was calculated by relating the areas of the observed resonances to the areas in the spectrum of a sample containing propionate, butyrate, and acetate (100 mM concentrations of each; 1.11% natural abundance) measured under identical conditions with dioxane as an internal standard.

Other analytical techniques. The remainder of the 3-ml samples withdrawn for NMR measurements was analyzed for organic acids. Also, 0.4-ml gas samples were withdrawn to determine the amount of CH_4 produced. Organic acids acids were measured with a Spectrasystem HPLC system equipped with an autosampler and Refractomonitor. The acids were separated on a Polyspher OAHY column (30 cm by 6.5 mm; Merck, Darmstadt, Germany) in 0.01 N H_2SO_4 at a flow rate of 0.6 ml/min and a column temperature of 60°C. The acids eluting from the column were quantified by differential refractometry (17).

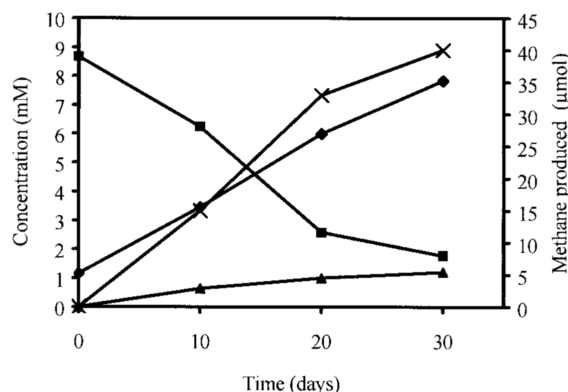

FIG. 1. Growth of *S. propionica* in coculture with *M. hungatei* JF1 in 50-ml batches. ■, Propionate; ◆, acetate; ▲, butyrate; ×, methane produced.

Methane levels were measured chromatographically with a Packard-Becker 417 gas chromatograph equipped with a thermal conductivity detector and molecular sieve 13X (60/80 mesh). The column temperature was 50°C, and the carrier gas was argon at a flow rate of 30 ml/min.

RESULTS

Growth experiments. Growth of the syntrophic coculture of *S. propionica* and *M. hungatei* is shown in Fig. 1. After 30 days of incubation, the culture produced 0.1 mol of methane, 1 mol of acetate, and 0.1 mol of butyrate per mol of propionate degraded (Fig. 1). In control bottles without propionate and in bottles to which 5 mM bromoethane sulfonate was added, no measurable changes in the organic acid concentration were observed and no methane was produced.

NMR measurements. When *S. propionica* was grown with [3-^{13}C]propionate, both [2-^{13}C]acetate and unlabeled acetate were produced, while [1-^{13}C]acetate was not formed (Tables 1 and 2). Label initially appeared mainly at the C4 position of butyrate, but after 30 days of incubation, label was recovered at the C2 and C4 positions of butyrate, in a ratio of about 1:1.5 (Table 1). [2-^{13}C]propionate yielded [1-^{13}C]acetate as well as unlabeled acetate, though small amounts of [2-^{13}C]acetate and [1,2-^{13}C]acetate were also detected. Throughout this experiment, nearly equal amounts of label were detected at the C1 and C3 positions of butyrate (Tables 1 and 2). The batches fed with [1-^{13}C]propionate yielded nearly equal amounts of [1-^{13}C]acetate, [2-^{13}C]acetate, and unlabeled acetate (Fig 2A; Tables 1 and 2). In this experiment the label initially appeared at the C2 position of butyrate, while after 30 days of incubation label was distributed more evenly over all carbon atoms (Fig. 2A). Double-labeled propionate at the C2 and C3 positions yielded [1-^{13}C]acetate, [2-^{13}C]acetate, and [1,2-^{13}C]acetate in a ratio near 1:1:1. Butyrate was initially mainly double labeled at the C3 and C4 positions, but after 30 days of incubation substantial amounts of single labeled butyrate and other combinations were also detected (Fig. 2B, Tables 1 and 2).

Label was also recovered in butyrate and propionate when the culture was grown on propionate in the presence of labeled acetate. Label from [1-^{13}C]acetate was detected at the C1 and C3 positions of butyrate and at the C2 of propionate (data not shown), whereas [2-^{13}C]acetate yielded label at the C2 and C4 positions of butyrate and at the methyl group of propionate (Table 1).

TABLE 1. ^{13}C recoveries from propionate conversion by *S. propionica* and *M. hungatei* after 30 days of incubation[a]

Isotope recovered	^{13}C recovery (%) with a substrate:				
	[1-^{13}C]P	[2-^{13}C]P	[3-^{13}C]P	[2,3-^{13}C]P	[2-^{13}C]A
[1-^{13}C]propionate	11				
[2-^{13}C]propionate	2	14		1	
[3-^{13}C]propionate	2		17	1	6
[1,2-^{13}C]propionate	1	2			
[2,3-^{13}C]propionate	1			15	
[1-^{13}C]acetate	29	53		16	
[2-^{13}C]acetate	27	1	62	18	57
[1,2-^{13}C]acetate	2	2		35	
[1-^{13}C]butyrate	4	9		3	
[1,2-^{13}C]butyrate				3	
[2-^{13}C]butyrate	5		9	1	9
[2,3-^{13}C]butyrate	2			3	
[3-^{13}C]butyrate	3	10		1	
[3,4-^{13}C]butyrate				5	
[4-^{13}C]butyrate	4		13	2	10
Total	93	91	101	104	82

[a] Propionate (P) was tested with label at the C1, C2, or C3 atom or double-labeled at the C2 and C3 atoms. Acetate (A) was introduced with label at the C2 atom. The inaccuracy of the quantified signals is usually $>5\%$ for methyl and methylene carbon atoms present at a concentration of at least 0.5 mM (values of $>10\%$ in the table). Quantification of signals present at lower concentrations and especially those of carboxylic acids is less accurate. However, the inaccuracy of those signals was usually only between 5 and 20%.

Although $H^{13}CO_3^{1-}$ was visible in all of the NMR spectra due to natural abundance (approximately 0.5 mM; Fig. 2), there were no substantial increases of the bicarbonate area observed. Therefore, we did not make further attempts to quantify $H^{13}CO_3^{1-}$ or $^{13}CH_4$. In addition, when the coculture was grown in the presence of 50 mM $H^{13}CO_3^{1-}$ we could not detect incorporation of label, since all the observed areas were due to the natural abundance of the compounds present, as was calculated from the high-pressure liquid chromatography data.

DISCUSSION

The stoichiometry of propionate conversion by the coculture of *S. propionica* and *M. hungatei* was similar, as reported previously (10). Our results obtained with ^{13}C-NMR support the theory that propionate is dismutated to acetate and butyrate, followed by syntrophic β-oxidation of butyrate to acetate. In addition, the results enabled us to propose a pathway of pro-

TABLE 2. Distribution of ^{13}C in acetate recovered from propionate conversion by *S. propionica* and *M. hungatei* after 30 days of incubation

Substrate	Mean concn (mM) of acetate isotope recovered			
	[1-^{13}C]	[2-^{13}C]	[1,2-^{13}C]	Unlabeled
[1-^{13}C]propionate	2.5 ± 0.2	2.3 ± 0.1	0.1 ± 0.03	2.1 ± 0.5
[2-^{13}C]propionate	4.7 ± 0.3	0.1 ± 0.03	0.2 ± 0.05	2.1 ± 0.5
[3-^{13}C]propionate	0	5.2 ± 0.2	0	1.7 ± 0.3
[2,3-^{13}C]propionate	2.3 ± 0.2	2.7 ± 0.1	2.6 ± 0.3	0

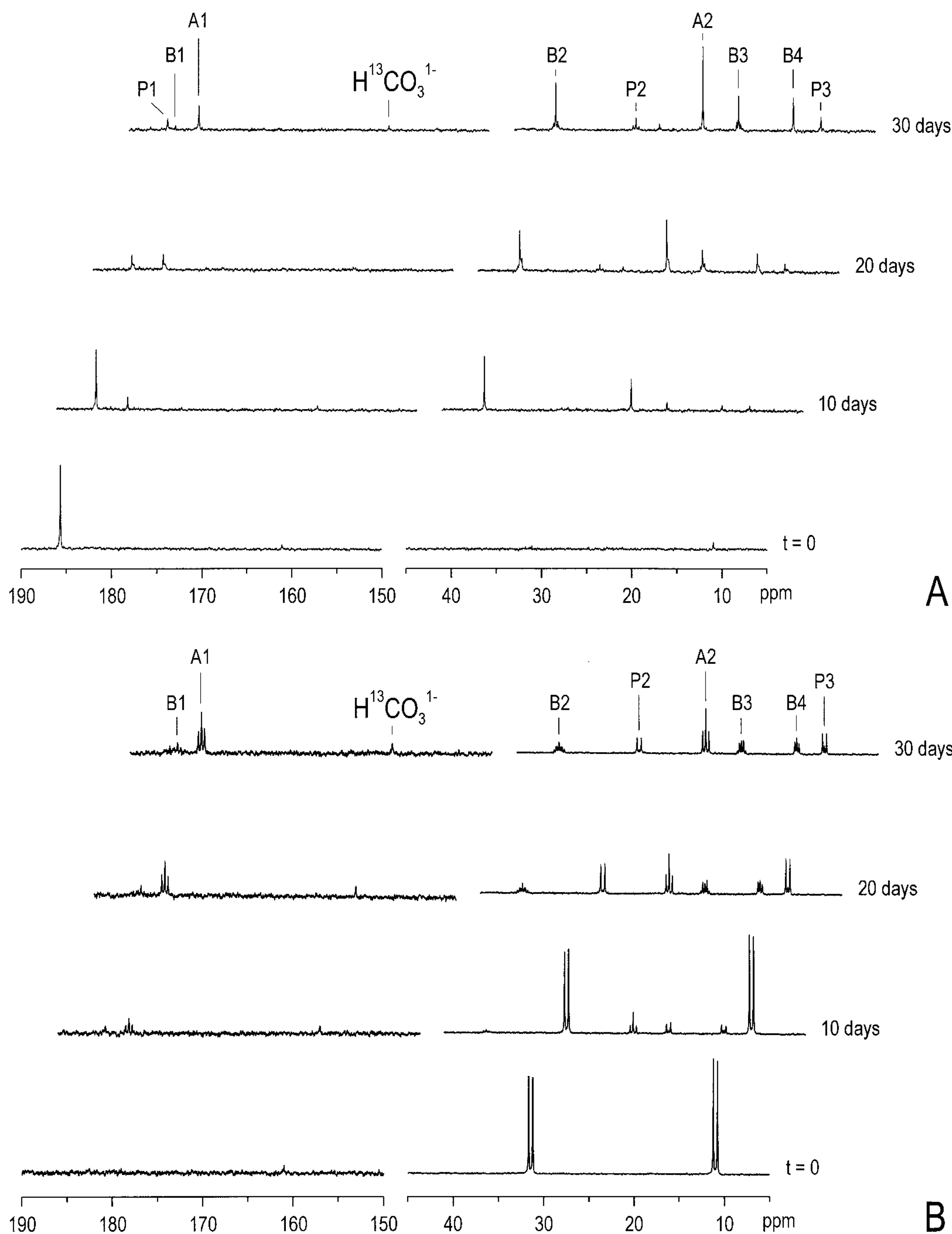

FIG. 2. Time courses of propionate conversion by *S. propionica* as measured by ^{1}H-decoupled ^{13}C-NMR. P, propionate; A, acetate; B, butyrate. The numbers refer to the position of the ^{13}C atoms. (A) Incubation with [1-^{13}C]propionate. (B) Incubation with [2,3-^{13}C]propionate. The resonances within the area of the carboxyl-groups (150 to 190 ppm) in this spectrum are enlarged by a factor 4.

pionate conversion by *S. propionica*. A randomizing pathway, which was found for several *Syntrophobacter* species, could be excluded since there was no exchange in label due to symmetry in any of the intermediates (6, 7, 11). Initially, we expected to find an acryloyl-CoA-like pathway in combination with reductive carboxylation, as reported in previous studies (9, 22, 23). However, [1-^{13}C]propionate did not yield $H^{13}CO_3^{1-}$, and experiments with [2,3-^{13}C]propionate showed that at least half of the methyl-methylene bonds were broken. Furthermore, $H^{13}CO_3^{1-}$ was not incorporated into propionate, indicating that the C1 of butyrate is introduced either via transcarboxylation or via Claisen condensation. Condensations involving propionyl-CoA were reported by Reeves and Ajl (12) and by Tabuchi et al. (20). However, these pathways both lead to the formation of acetyl-CoA via decarboxylation (of pyruvate) and do not explain the breakage of the methyl-methylene bonds either. Incubations with labeled acetate showed that an acetyl-CoA condensation pathway is present in *S. propionica*,

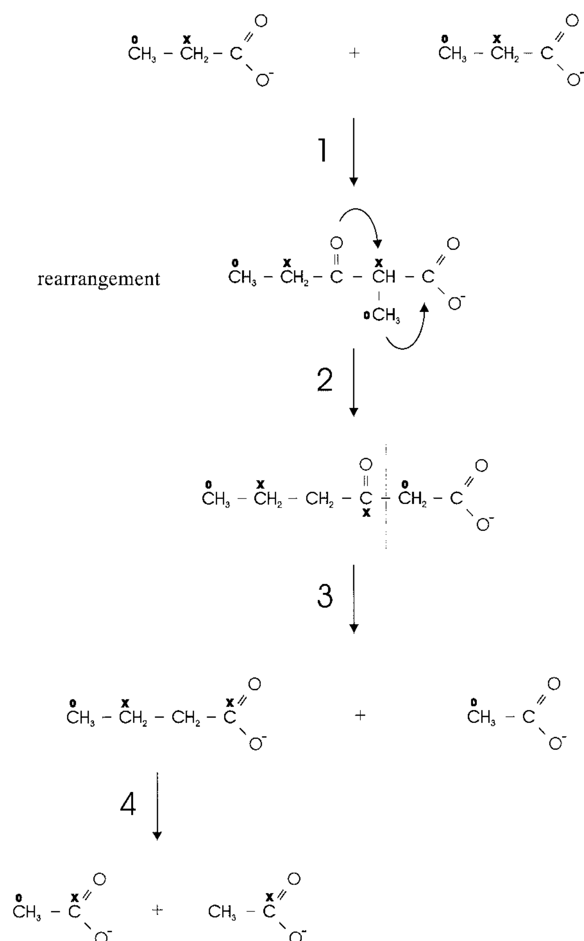

FIG. 3. Proposed pathway for propionate conversion by *S. propionica*. Step 1, condensation of the C2 of propionate to the carboxyl of another propionate molecule or derivative; step 2, rearrangement of the methyl group and transfer of the oxygen to the C3 of the intermediate; step 3, cleavage of 3-ketohexanoate yielding butyrate and acetate; step 4, syntrophic β-oxidation of butyrate to acetate.

most likely one similar to the pathway found for *Syntrophomonas wolfei* (24). However, the majority of the butyrate is produced in a different fashion, since single-labeled propionate initially yielded mainly single-labeled butyrate and [2,3-^{13}C]propionate initially yielded mainly [3,4-^{13}C]butyrate.

A pathway which could explain the observed labelling pattern is depicted in Fig. 3. The high levels of [1-^{13}C]butyrate from [2-^{13}C]propionate and [2,3-^{13}C]propionate suggest that the C2 of propionate is coupled to the carboxyl group of a second propionate molecule. A rearrangement of the six-carbon intermediate to give an unbranched molecule followed by cleavage of acetate would explain the ratios of labeled acetate, as well as the ratios of labeled to unlabeled acetate (Fig. 3). The residual four-carbon molecule (butyrate) is then further oxidized syntrophically to acetate, a result which agrees with the amounts of methane produced. The presence of such pathway is strongly favored by the fact that we could not demonstrate incorporation or excretion of $H^{13}CO_3^{1-}$. The incubations in the presence of labeled acetate revealed that the pathway is reversible. This explains the observed shift of label in time toward a more equal distribution in butyrate. It also explains why small amounts of label are recovered in [2-^{13}C]acetate from [2-^{13}C]propionate and double-labeled acetate from either [1-^{13}C]- or [2-^{13}C]propionate, while [3-^{13}C]propionate yielded exclusively [2-^{13}C]acetate. In addition, it explains the distribution of label in butyrate, as well as the formation of [2,3-^{13}C]butyrate from [2-^{13}C]propionate.

Most likely all steps in the proposed pathway require CoA derivatives, as occurs during butyrate oxidation. The initial activation of propionate may be accomplished by CoA transfer from acetyl-CoA or another CoA-containing intermediate. Like other rearrangement reactions, the isomerization of the two-methyl group to an unbranched molecule is likely a coenzyme B_{12}-dependent reaction. The mechanism of this rearrangement may be identical to the reaction catalyzed by methyl-malonyl-CoA mutase (4). Transfer of the keto group would require a reduction and a dehydration, yielding a double bond between C3 and C4, followed by the addition of H_2O and oxidation to 3-ketohexanoate. Possibly the last two steps are catalyzed by crotonase and butyryl-CoA dehydrogenase, enzymes also required for the cleavage of butyrate.

S. propionica is the first syntrophic propionate-oxidizer that may account for the nonrandomizing pathway observed in methanogenic habitats. The results seem to fit in previous studies with methanogenic biomass and enrichment cultures in which the presence of an alternative route for propionate oxidation was clearly demonstrated by the use of ^{13}C-NMR (9, 22, 23). The isolation of *S. propionica* enabled us to study this pathway into detail without interference of the randomizing methyl-malonyl-CoA pathway which also occurs in complex microbial communities. It might be interesting to study the occurrence of *S. propionica* or microorganisms with a similar pathway in anaerobic digesters and other methanogenic environments.

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