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 13C-NMR spectroscopy. Cocultures produced acetate and butyrate from propionate. [3-13C]propionate was converted to [2-13C]acetate, with no [1-13C]acetate formed. Butyrate from [3-13C]propionate was labeled at the C2 and C4 positions in a ratio of about 1:1.5. Double-labeled propionate (2,3-13C) yielded not only double-labeled acetate but also single-labeled acetate at the C1 or C2 position. Most butyrate formed from [2,3-13C]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. 1-13C-labeled propionate yielded both [1-13C]acetate and [2-13C]acetate. When 13C-labeled bicarbonate was present, label was not incorporated into acetate, propionate, or butyrate. In each of the incubations described above, 13C was never recovered in bicarbonate or methane. These results indicate that S. propionica does not degrade propionate via the methylmalonyl-coenzyme A (CoA) pathway or any other of the known pathways, such as the acyl-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 (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22). However, other pathways of propionate degradation are possible as well, such as a nonrandomizing pathway via butyrate (8, 13, 14, 15, 22).

MATERIALS AND METHODS

Organisms and cultivation. Methanospirillum hungatei JF1 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 mercaptoethanol sulfonate, was used to grow syntrophic cultures of S. propionica and M. hungatei. The methanogens were pregrown on H2 and CO2 in 120-ml serum vials with 50 ml of medium. After growth the gas atmosphere was replaced by N2 and CO2 (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% 13C) were obtained from Campro Scientific B.V. (Veenendaal, The Netherlands). Serum vials were prepared with 10 mM concentrations of either [1-13C]propionate, [2-13C]propionate, [3-13C]propionate, or [2,3-13C]propionate as substrates in 50 ml of medium. To test the incorporation of H13CO3−, the coculture was grown on 10 mM unlabeled propionate in the presence of 50 mM H13CO3−. The combination of 10 mM unlabeled propionate and 4 mM [1-13C]acetate or [2-13C]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 D2O 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% D2O and 100 mM dioxane. The proton-decoupled 13C-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 deuteration in the samples (10% [vol/vol]) was used for the field lock. A balance of 13C-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 CH4 produced. Organic acids were measured with a Spectrasonik HPLC system equipped with an autosampler and Refractometer Monitor. The acids were separated on a Polyspher OAHY column (30 cm by 6.5 mm; Merck, Darmstadt, Germany) in 0.01 N H2SO4 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).
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 concentrations were observed and no methane was produced.

NMR measurements. When S. propionica was grown with [3-13C]propionate, both [2-13C]acetate and unlabeled acetate were produced, while [1-13C]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 and at the methyl group of propionate (Table 1).

Although H13CO3− 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 due to the natural abundance of the compounds present, as reported previously (10). Our results obtained with 13C-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 propionate conversion by S. propionica and M. hungatei after 30 days of incubation.

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 13C-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 propionate conversion by S. propionica and M. hungatei after 30 days of incubation.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Mean concn (mM) of acetate isotope recovered</th>
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<tbody>
<tr>
<td>[1-13C]propionate</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>[2,3-13C]propionate</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>[3-13C]propionate</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>[2,3-13C]propionate</td>
<td>2.3 ± 0.2</td>
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TABLE 2. Distribution of 13C in acetate recovered from propionate conversion by S. propionica and M. hungatei after 30 days of incubation

FIG. 1. Growth of S. propionica in coculture with M. hungatei JF1 in 50-ml batches. ■ Propionate; ○ acetate; ▲ butyrate; × methane produced.
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, [1-13C]propionate did not yield H\(^{13}\text{CO}_3\)\(^{1-}\), and experiments with [2,3-13C]propionate showed that at least half of the methyl-methylene bonds were broken. Furthermore, H\(^{13}\text{CO}_3\)\(^{1-}\) was not incorporated into propionate, indicating that the C1 of butyrate is introduced either via trans-carboxylation 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. 2. Time courses of propionate conversion by *S. propionica* as measured by \(^1\text{H}-\text{decoupled}^{13}\text{C-NMR. P, propionate; A, acetate; B, butyrate. The numbers refer to the position of the }^{13}\text{C atoms. (A) Incubation with [1-}^{13}\text{C}]propionate. (B) Incubation with [2,3-}^{13}\text{C}]propionate. The resonances within the area of the carboxyl-groups (150 to 190 ppm) in this spectrum are enlarged by a factor 4.}
from $\text{[2-}^{13}\text{C}]$propionate and $\text{[2,3-}^{13}\text{C}]$propionate suggest that
tern is depicted in Fig. 3. The high levels of $\text{[1-}^{13}\text{C}]$butyrate
initially yielded mainly single-labeled butyrate and $\text{[2,3-}^{13}\text{C}]$propionate initially yielded mainly $\text{[3,4-}^{13}\text{C}]$butyrate. (24). However, the majority of the butyrate is pro-

Syntrophomonas wolfei

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 $\text{[2,3-}^{13}\text{C}]$propionate initially yielded mainly $\text{[3,4-}^{13}\text{C}]$butyrate.

A pathway which could explain the observed labelling pattern is depicted in Fig. 3. The high levels of $\text{[1,3}^{13}\text{C}]$butyrate from $\text{[2-}^{13}\text{C}]$propionate and $\text{[2,3-}^{13}\text{C}]$propionate suggest that the C2 of propionate is coupled to the carboxyl 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 (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 in anaerobic digesters and other methanogenic environments.

REFERENCES


2. Boone, D. R. 1984. Propionate exchange reactions in methanogenic ecosys-


