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Pathway of Butyrate Catabolism by *Desulfobacterium cetonicum*

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***Desulfobacterium cetonicum* 480 oxidized butyrate to 1 mol of acetate and 2 mol of CO_2; this reaction was coupled to reduction of sulfate to sulfide. Butyrate was activated by coenzyme A (CoA) transfer from acetyl-CoA, and butyryl-CoA was oxidized to acetyl-CoA by a classical β-oxidation pathway. Acetyl-CoA was oxidized through the acetyl-CoA/carbon monoxide dehydrogenase pathway. There was a rapid exchange of $^{14}CO_2$ into the intermediate CoA esters and into acetate and butyrate, showing that all of the steps involved in the oxidation of butyrate to acetyl-CoA are reversible.**

Since the discovery of many new types of sulfate-reducing bacteria in the last 15 years, the carbon metabolism of these organisms has been investigated in some detail, particularly the different pathways of acetyl coenzyme A (acetyl-CoA) oxidation to CO_2 (14, 17). The utilization of fatty acids by sulfate-reducing bacteria is postulated to proceed via β-oxidation and the activation of fatty acids by CoA transfer from acetyl-CoA (12), although this has not been shown (17). We have investigated the catabolism of butyrate in the sulfate-reducing bacterium *Desulfobacterium cetonicum.*

Growth on butyrate. *D. cetonicum* 480 (6) was obtained from A. S. Galushko (Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia). Cultures were grown in the dark at 30°C in 200-ml aliquots in 250-ml glass bottles sealed with black rubber stoppers and with a headspace of N_2 plus CO_2 (80:20, vol/vol). A sulfide-reduced bicarbonate-buffered mineral medium supplemented with a seven-vitamin mixture (4) and with the NaCl and $MgCl_2 \cdot 6H_2O$ concentrations increased to 10 and 1.75 g · liter^{-1}, respectively, was used with 10 mM butyrate as the carbon and energy source and 20 mM Na_2SO_4 as the electron acceptor. Growth and butyrate catabolism were monitored in two cultures (means of data are presented). Acetate and butyrate were measured by high-pressure liquid chromatography (HPLC) with a refractive index detector (5). Sulfide was measured by a microassay modification of the method of Trüper and Schlegel (16). Cell densities in cultures and cell suspensions were measured as optical densities in 10-mm cuvettes at 440 nm and were correlated to gravimetrically determined dry mass and total protein by using data obtained from similarly grown cultures.

Acetate (5.4 mmol · liter^{-1}) and sulfide (14.4 mmol · liter^{-1}) were produced concomitantly with butyrate utilization (8.4 mmol · liter^{-1}) and an increase in the culture density of 38.4 mg (dry cell mass) · liter^{-1} (Fig. 1). The doubling time was 38.6 h (growth rate = 0.432 day^{-1}). Approximately 1 mol of acetate was produced per mol of butyrate degraded in the first 7 days, in agreement with the following equation ($\Delta G_0'$ calculated from the data of Thauer et al. [15]): butyrate$^-$ + 1 1/2SO_4^{2-} → acetate$^-$ + 2 HCO_3^- + 1 1/2HS^- + 1/2H^+; $\Delta G_0' = -75.15$ kJ · reaction^{-1}. The expected sulfide production was 15.6 mmol · liter^{-1}, and thus the sulfide recovery was 92%. The specific growth yield in batch culture was 4.6 g (dry mass) · mol of butyrate^{-1}. The cellular protein content was 0.508 g of protein · g (dry weight)$^{-1}$. From the specific growth rate and specific growth yield, the in vivo substrate turnover rate was calculated to be 129 nmol · min^{-1} · mg of protein^{-1}. Once butyrate was almost exhausted, acetate was slowly reutilized.

Enzymology of butyrate degradation. Butyrate-grown cells were harvested by centrifugation under strictly anoxic conditions as previously described (9) and were washed and resuspended in anoxic 125 mM triethanolamine · HCl (pH 7.4) containing 2.5 mM dithioerythritol. Cell extracts were prepared by French press treatment as described elsewhere (9), and enzyme activities were measured (Table 1). Butyrate was activated by CoA transfer from acetyl-CoA. No butyryl-CoA synthetase could be detected. Butyryl-CoA was oxidized to crotonyl-CoA; we used dichlorophenol indophenol as an artificial electron acceptor in the enzyme assay. High levels of 3-enoyl-CoA hydratase (crotonase) activity were measured. 3-Hydroxybutyryl-CoA was oxidized to acetoacetyl-CoA with NAD as the acceptor; NADP could not substitute for NAD. Acetoacetyl-CoA was thiolytically cleaved to two acetyl-CoA moieties by an acetyl-CoA acetyltransferase (3-ketothiolase). Enzymes of the acetyl-CoA/carbon monoxide dehydrogenase

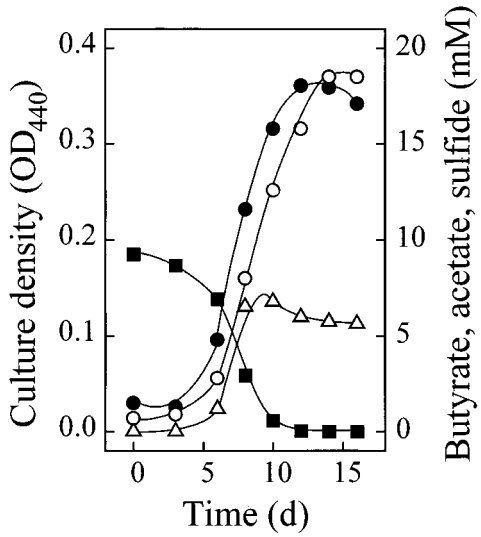

FIG. 1. Growth of *D. cetonicum* on butyrate plus sulfate, showing the utilization of butyrate (■) and the concomitant production of sulfide (○), acetate (△), and cell matter (●) (culture density measured as optical density at 440 nm [OD_{440}]).

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TABLE 1. Enzyme activities measured in crude cell extracts of *D. cetonicum* grown with butyrate plus sulfate[a]

Enzyme (acceptor)	EC no.	Assay method (reference)	Sp act (μmol · min^{-1} · mg of protein^{-1})[b]
Butyryl-CoA:acetate CoA transferase	2.8.3.8	18	0.143–0.191
Butyryl-CoA synthetase	6.2.1.2	10[c]	<0.001
Acyl-CoA dehydrogenase (dichlorophenol indophenol)	1.3.99.3	18[d]	1.67–2.17
3-Enoyl-CoA hydratase	42.1.17/55	18[e]	37.4–42.1
3-Hydroxybutyryl-CoA dehydrogenase (NAD)	1.1.1.35	11	2.40–2.72
3-Hydroxybutyryl-CoA dehydrogenase (NADP)	1.1.1.36	11	<0.001
3-Hydroxybutyrate dehydrogenase (NAD or NADP)	1.1.1.30	11	<0.001
Acetyl-CoA acetyltransferase	2.3.1.9	18	1.67–2.17
Carbon monoxide dehydrogenase (methylviologen)	1.2.99.2	3	0.91–1.03
Formate dehydrogenase (methylviologen)	1.2.99.–	3[f]	1.72–2.96

[a] Assays were carried out at 30°C as described by Brune and Schink (2). The reduction of 2 μmol of methylviologen was defined as the oxidation of 1 μmol of substrate. Protein was quantified by the microassay of Bradford (1) with bovine serum albumin as a standard.
[b] Means of three to five determinations.
[c] Modified by starting with butyrate instead of acetate.
[d] With butyryl-CoA as the substrate.
[e] Modified by omitting bovine serum albumin and increasing [NAD^+] to 2 mM.
[f] Except under N_2 with the addition of 10 mM Na formate.

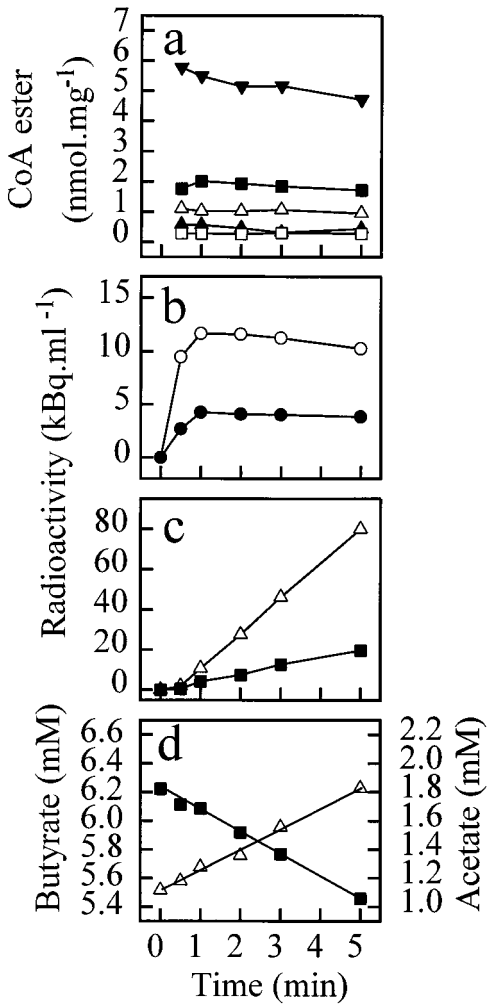

FIG. 2. (a) Intracellular CoA ester levels in butyrate-grown cells of *D. cetonicum* during butyrate degradation by a dense cell suspension. Butyrate was added 1 min before time zero. ▼, 3-hydroxybutyryl-CoA (mean level, 5.26 nmol · mg of protein^{-1}); ■, butyryl-CoA (mean level, 1.86 nmol · mg of protein^{-1}); △, acetyl-CoA (mean level, 1.03 nmol · mg of protein^{-1}); ▲, acetoacetyl-CoA (mean level, 0.50 nmol · mg of protein^{-1}); □, crotonyl-CoA (mean level, 0.29 nmol · mg of protein^{-1}). (b) Incorporation of ^{14}C into CoA esters. $^{14}CO_2$ was added at time zero. ○, radioactivity in pool 1 (3-hydroxybutyryl-CoA plus acetoacetyl-CoA plus acetyl-CoA); ●, radioactivity in pool 2 (butyryl-CoA plus crotonyl-CoA). (c and d) Incorporation of ^{14}C into acetate (△) and butyrate (■) (c) and formation of acetate (△) and utilization of butyrate (■) (d) in the same experiment.

pathway were also present, as indicated by the activities of formate dehydrogenase and carbon monoxide dehydrogenase (both measured with oxidized methylviologen as an artificial electron acceptor).

$^{14}CO_2$ exchange into CoA-esters and free fatty acids. Dense suspensions (1 ml) of butyrate-grown cells (6.5 mg of protein · ml^{-1}) were prepared by centrifugation under anoxic conditions and were washed and resuspended in 50 mM potassium phosphate (pH 7.2) supplemented with 20 mM Na_2SO_4 and 2.5 mM dithioerythritol and containing 10.0 g of NaCl and 1.75 g of $MgCl_2 \cdot 6H_2O$ per liter. The suspensions were incubated at 30°C with 30 mM $NaHCO_3$ under a headspace of N_2 plus CO_2 (80:20, vol/vol). Butyrate (5 mM) was added, and after 1 min, 500 kBq of $Na_2{}^{14}CO_3$ in 20 μl of 1 mM KOH was added. Samples of 200 μl were taken, added to 25 μl of 70% (wt/vol) perchloric acid, allowed to stand for 30 min, centrifuged in a bench top microcentrifuge at 5,000 × *g*, bubbled with CO_2 at 10 ml · min^{-1} for 30 min, and then centrifuged again. The CoA esters were separated by reversed-phase HPLC (2, 7) connected to a RAMONA-5 radioactivity HPLC monitor analyzer (Raytest, Straubenhardt, Germany) fitted with a 400-μl glass scintillator flow cell and were quantified by comparison with standards of CoA esters also treated with perchloric acid. Perchloric acid-treated CoA esters were stable. The radioactivity analyzer was calibrated with [1-^{14}C]benzoate. Acetyl-CoA, acetoacetyl-CoA, and 3-hydroxybutyryl-CoA eluted close together as three sharply defined peaks when measured with a UV detector at 206 nm, but only one broad peak of radioactivity (pool 1) covering all three CoA esters could be measured. Similarly, the radioactivities in butyryl-CoA and crotonyl-CoA (pool 2) could not be separated, although the CoA esters were clearly baseline separated when detected with a UV detector. The radioactivities in acetate, butyrate, pool 1, and pool 2 were well separated by these methods.

In butyrate-degrading cell suspensions of butyrate-grown *D. cetonicum*, the intermediate CoA esters suggested by the measured enzyme activities could be detected (Fig. 2a). Ninety seconds after the addition of butyrate, the levels of these CoA esters were constant, except for that of 3-hydroxybutyryl-CoA, which fell slightly over the 5-min experiment. Addition of $^{14}CO_2$ to butyrate-degrading cell suspensions of butyrate-grown *D. cetonicum* resulted in incorporation of the radiolabel into intracellular CoA esters. Both pools rapidly accumulated label over the first minute of the experiment, and then the

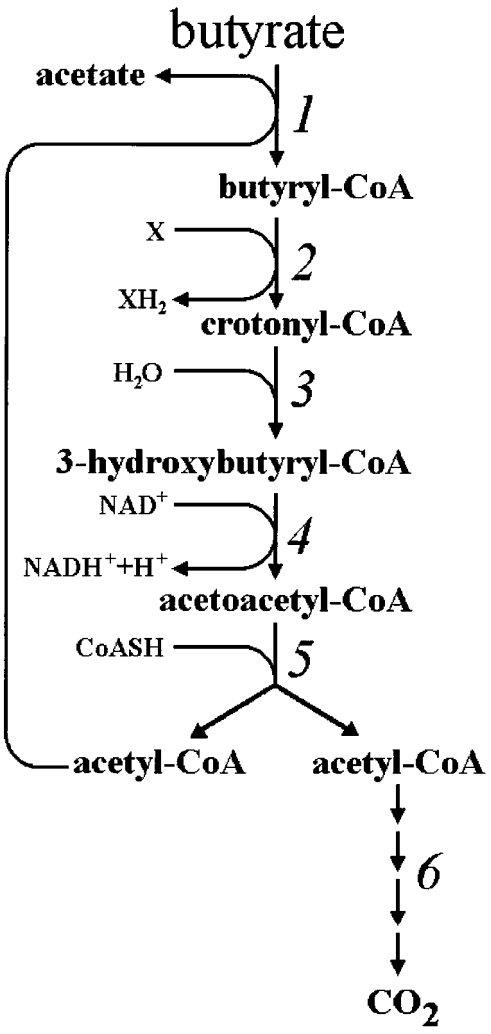

FIG. 3. Proposed pathway of butyrate degradation by *D. cetonicum*. All reactions are reversible, as shown by the rapid accumulation of radiolabel from $^{14}CO_2$ into butyrate. The enzymes involved are as follows: *1*, butyryl-CoA:acetate CoA transferase; *2*, acyl-CoA dehydrogenase; *3*, 3-enoyl-CoA hydratase; *4*, 3-hydroxybutyryl-CoA dehydrogenase (NAD dependent); *5*, acetyl-CoA acetyltransferase; and *6*, enzymes of the acetyl-CoA/carbon monoxide dehydrogenase pathway. X denotes the unknown acceptor for the acyl-CoA dehydrogenase; we used dichlorophenol indophenol in the assays.

specific label content remained constant (Fig. 2b), indicating that the rate of new label entering the CoA ester pools was equal to the rate of label leaving the pools as CO_2, acetate, and butyrate.

The addition of $^{14}CO_2$ to cell suspensions degrading butyrate resulted in a rapid labelling of acetate and butyrate (Fig. 2c). The kinetics of label incorporation was linear for both acids, after an initial lag period of about 30 s. The apparent exchange activities of CO_2 into the free fatty acids were 23.4 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ for acetate and 5.7 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ for butyrate. In the same experiments, the cell suspensions degraded butyrate at a rate of 20.2 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ and produced acetate at a rate of 21.5 $nmol \cdot min^{-1} \cdot mg$ of $protein^{-1}$ (Fig. 2d). The labelling can be explained by the exchange of CO_2 into acetyl-CoA catalyzed by the enzymes of the acetyl-CoA/carbon monoxide dehydrogenase pathway (12, 14). The rate of CO_2 (total pool) exchange into butyrate, once a constant rate was obtained, was 28% of the butyrate degradation rate, and that of CO_2 exchange into acetate was 109% of the acetate production rate. Thus, radiolabel is incorporated into acetyl-CoA and then flows back through the β-oxidation pathway to appear as labelled butyrate. These data show that the reactions from butyrate to acetyl-CoA are all reversible.

The proposed β-oxidation pathway of butyrate degradation, consistent with the enzyme activities and substrate/product balances measured, is shown in Fig. 3. The CoA esters detected in butyrate-degrading cells of *D. cetonicum* were also consistent with the proposed pathway. All of the enzyme activities involved in butyrate oxidation to acetyl-CoA were present at levels high enough to account for the in vivo rate of turnover of butyrate by growing cultures. As the butyrate concentration decreased in the batch culture, acetate utilization was initiated, which was seen as a decrease in the acetate concentration (Fig. 1). Under butyrate-sufficient conditions, the ratio of acetate produced to butyrate utilized was nearly 1:1 (Fig. 2d). Acetate supports growth of *D. cetonicum* (6). The presence of an acetyl-CoA synthetase was suggested by enzyme activity measurements (8), but this was not investigated further. As has been pointed out previously (13), the mechanism of acetate activation in sulfate-reducing bacteria warrants further investigation.

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