APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1992, p. 1435–1439
0099-2240/92/051435-05$02.00/0

Vol. 58, No. 5

Reciprocal Isomerization of Butyrate and Isobutyrate by the Strictly Anaerobic Bacterium Strain WoG13 and Methanogenic Isobutyrate Degradation by a Defined Triculture

CAROLA MATTHIES† AND BERNHARD SCHINK‡*

Lehrstuhl Mikrobiologie I, Eberhard-Karls-Universität, Auf der Morgenstelle 28, D-7400 Tübingen, Germany

Received 14 November 1991/Accepted 12 February 1992

Isomerization of butyrate and isobutyrate was investigated with the recently isolated strictly anaerobic bacterium strain WoG13 which ferments glutarate to butyrate, isobutyrate, CO_2, and small amounts of acetate. Dense cell suspensions converted butyrate to isobutyrate and isobutyrate to butyrate. ^{13}C-nuclear magnetic resonance experiments proved that this isomerization was accomplished by migration of the carboxyl group to the adjacent carbon atom. In cell extracts, both butyrate and isobutyrate were activated to their coenzyme A (CoA) esters by acyl-CoA:acetate CoA-transferases. The reciprocal rearrangement of butyryl-CoA and isobutyryl-CoA was catalyzed by a butyryl-CoA:isobutyryl-CoA mutase which depended strictly on the presence of coenzyme B_{12}. Isobutyrate was completely degraded via butyrate to acetate and methane by a defined triculture of strain WoG13, *Syntrophomonas wolfei*, and *Methanospirillum hungatei*.

A strictly anaerobic bacterium, strain WoG13, growing with dicarboxylic acid glutarate as the energy source was recently isolated from anoxic sediment samples (8). Strain WoG13 fermented glutarate to CO_2 plus a mixture of butyrate and isobutyrate at a ratio of nearly 1:1. In addition, small amounts of acetate were formed. This butyrate isomerization reaction is of major interest in anaerobic metabolism.

Conversion of butyrate to isobutyrate was observed in lake sediment samples if butyrate degradation was inhibited by added hydrogen (7). ^{13}C-nuclear magnetic resonance (^{13}C-NMR) studies in cultures enriched with isobutyrate from methanogenic digester sludge demonstrated that rearrangement of the carbon skeleton could be characterized as migration of the butyrate carboxyl group from C-2 to C-3 to form isobutyrate (14). Isobutyrate degradation proceeds via two different pathways under anaerobic conditions. With sulfate as the terminal electron acceptor, isobutyrate was degraded by β-oxidation and was further metabolized via methylmalonyl-coenzyme A (methylmalonyl-CoA) and the CO dehydrogenase pathway (12). Methanogenic degradation of isobutyrate was shown in nondefined microbial communities involving intermediate formation of butyrate and further oxidation of butyrate to acetate and methane (12).

In the aerobic bacterium *Streptomyces cinnamonensis*, interconversion of isobutyrate to butyrate is a key reaction in the biosynthesis of the polyether antibiotic monensin-A (5, 10, 11). In this bacterium, a coenzyme B_{12}-dependent isobutyryl-CoA:butyryl-CoA mutase catalyzes the isomerization (3).

In the present study, reciprocal isomerization of butyrate and isobutyrate is described for the first time in a pure culture of a strictly anaerobic bacterium. The enzymes involved in isomerization are characterized in cell extracts. In addition, complete isobutyrate degradation to acetate and methane by a defined triculture is shown.

* Corresponding author.
† Present address: BITÖK, Universität Bayreuth, D-8580 Bayreuth, Germany.
‡ Present address: Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-7750 Konstanz, Germany.

MATERIALS AND METHODS

Sources of organisms. Strain WoG13 was isolated in pure culture from anoxic mud of a polluted ditch near Konstanz, Germany (8). *Syntrophomonas wolfei* in coculture with *Methanospirillum hungatei* (DSM 2245B) was obtained from the Deutsche Sammlung von Mikroorganismen GmbH (Braunschweig, Germany).

Medium and growth conditions. All bacteria used were cultivated in a carbonate-buffered (30 mM), sulfide-reduced (1 mM) freshwater mineral salts medium (a modification of that described in reference 17). The medium contained 1 ml of trace element solution SL 10 per liter, 1 ml of selenite-tungstate solution (16) per liter, and a seven-vitamin solution (17). For strain WoG13, the medium was supplemented with 2% (vol/vol) rumen fluid (8) and 30 mM glutarate as the growth substrate. For cell suspension experiments and enzyme assays, strain WoG13 was grown in 125-ml infusion flasks which were filled with 100 ml of medium, gassed with a gas mixture of N_2-CO_2 (90%/10%), and sealed with butyl rubber stoppers. The coculture of *S. wolfei-M. hungatei* was cultivated with 20 mM butyrate and 0.05% (wt/vol) yeast extract. All cultures were incubated at 30°C.

Preparation of cell suspensions and cell extracts. Cultures of strain WoG13 (400 ml) at the late logarithmic phase were centrifuged in the infusion flasks at 3,500 × *g* for 30 min. If not indicated otherwise, cells were resuspended in potassium phosphate buffer (50 mM, pH 7.0, reduced with 2.5 mM dithioerythritol) and transferred into an 8-ml serum bottle, which was gassed with N_2 and sealed with a butyl rubber stopper. Cells were washed twice by centrifugation and resuspension in the same buffer and concentrated to an optical density at 450 nm (OD_{450}) of 5 to 10 (corresponding to a protein content of 0.6 to 1.2 mg liter^{-1}).

One-liter cultures of the coculture *S. wolfei-M. hungatei* were harvested in a glove box and centrifuged at 13,500 × *g*

for 20 min, and the cells were resuspended and washed with freshwater medium under anoxic conditions.

For enzyme assays, crude cell extracts of strain WoG13 were prepared from high-density cell suspensions by treatment with a French press at 6.9 MPa under anoxic conditions (N_2 atmosphere). Unbroken cells were sedimented by centrifugation (4,500 × *g*, 15 min), and the clear supernatant was used for enzyme assays.

Experiments with resting cell suspensions. Isomerization was investigated in 5- or 8-ml vials filled with cell suspensions (OD_{450} = 5 to 10), gassed with N_2, and sealed with butyl rubber stoppers. The reaction was started by injecting either sodium butyrate or sodium isobutyrate to a concentration of 10 mM. Samples taken by syringes were immediately centrifuged and frozen at −20°C. The samples were analyzed by gas chromatography (see below).

Triculture experiments were carried out in 25-ml serum bottles which were filled with 2-ml cell suspensions prepared with freshwater medium and gassed with 90% N_2–10% CO_2.

Enzyme assays. (i) Mutase assay. Butyryl-CoA:isobutyryl-CoA mutase activity was measured in cell extracts of strain WoG13 by high-pressure liquid chromatography (HPLC) analysis of the decrease or increase of butyryl-CoA and isobutyryl-CoA. The assay mixture contained potassium phosphate buffer (50 mM [pH 7.0] plus 2.5 mM dithioerythritol), adenosyl cobalamine (20 μM), butyryl-CoA or isobutyryl-CoA (1 mM), and 0.4 mg of cell protein. Assay vials were incubated in the dark at 25°C. Samples (0.1 ml) were taken with syringes and injected into 0.4 ml of H_3PO_4 (0.1 M) to stop the reaction.

(ii) Acyl-CoA:acetate CoA-transferase assay. Acyl-CoA:acetate CoA-transferase activities were determined photometrically in a spectrophotometer (model 100-40; Hitachi, Tokyo, Japan) under anoxic conditions. The coupled assay (6) contained sodium arsenate buffer (10 mM [pH 7.0]), KCl (50 mM), phosphate acetyl transferase (0.4 U/ml, from *Bacillus stearothermophilus*), sodium acetate (1 mM), butyryl-CoA or isobutyryl-CoA (0.1 mM), and 0.01 to 0.02 mg of protein ml^{-1}.

Chemical analyses. Butyrate, isobutyrate, and acetate were determined by gas chromatography (9). Injector and detector temperatures were both 200°C; the column temperature was 150°C. Methane was measured with an ML GC 82-11 gas chromatograph (Mikrolab, Højbjerg, Denmark) with flame ionization detection (120°C). A column filled with Porapak N (Waters, Milford, Mass.) was used at a temperature of 80°C with N_2 as the carrier gas (150 ml min^{-1}).

Coenzyme A esters of butyrate and isobutyrate were quantified with a Beckman System Gold HPLC. A column (4 by 250 mm) filled with LiChrospher 100 RP-18 endcapped (5 μm) (Merck, Darmstadt, Germany) was used. Elution of samples (20 μl) was carried out with a potassium phosphate buffer (50 mM, pH 6.5)-methanol solvent system at a flow rate of 1 ml/min. Butyryl-CoA and isobutyryl-CoA were separated with a linear methanol gradient (26 to 29%) increasing over 8 min. The CoA esters were detected in a Beckman 167 or 166 variable-wavelength detector at 260 nm. The chromatograms were analyzed by a computer program, and the peaks were identified and quantified by comparison with external standards.

Protein in cell extracts and cell suspensions was determined by dye formation with $CuSO_4$ in alkaline solution (a modification of that described in reference 18).

^{13}C-NMR spectroscopy. ^{13}C-NMR spectroscopy was carried out at the Institut National de la Recherche Agronomique (Villeneuve d'Ascq, France) as described previously (15).

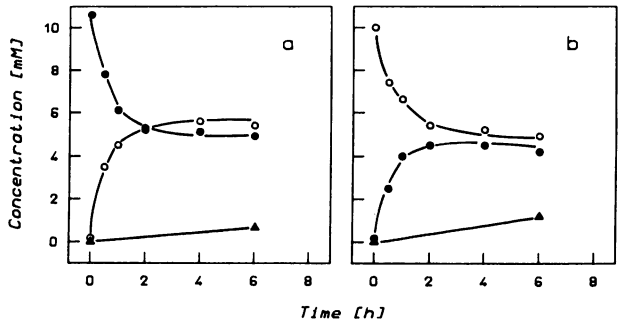

FIG. 1. Reciprocal rearrangement of butyrate and isobutyrate in dense cell suspensions of strain WoG13. The assay mixture contained 2 mg of protein ml^{-1}. (a) Butyrate as substrate; (b) isobutyrate as substrate. Symbols: ●, butyrate; ○, isobutyrate; ▲, acetate.

Chemicals. All chemicals used were of analytical grade quality and obtained from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Sigma (Deisenhofen, Germany), and Boehringer (Mannheim, Germany). Gases were obtained from Messer-Griesheim, Darmstadt, Germany.

RESULTS

Isomerization of butyrate and isobutyrate by resting cells. Dense cell suspensions (OD_{450} = 9.5) of strain WoG13 were incubated with either butyrate or isobutyrate. As shown in Fig. 1, the cells converted butyrate to isobutyrate and vice versa. The formation rates of the rearranged fatty acids were 84 and 47 nmol/min · mg of $protein^{-1}$ for butyrate-to-isobutyrate and isobutyrate-to-butyrate conversions, respectively. Isomerization reached an equilibrium after about 4 h. The equilibrium concentrations represented an isobutyrate-to-butyrate ratio of 1.1:1 or 1.2:1, respectively. In addition, small amounts of acetate were formed (Fig. 1). Carbon recoveries were 97% (Fig. 1a) and 95% (Fig. 1b).

Experiments with cell extracts. Cell extracts of strain WoG13 catalyzed the isomerization of butyryl-CoA to isobutyryl-CoA or vice versa (Fig. 2). With butyryl-CoA as the substrate, an activity of 19 nmol/min · mg of $protein^{-1}$ was

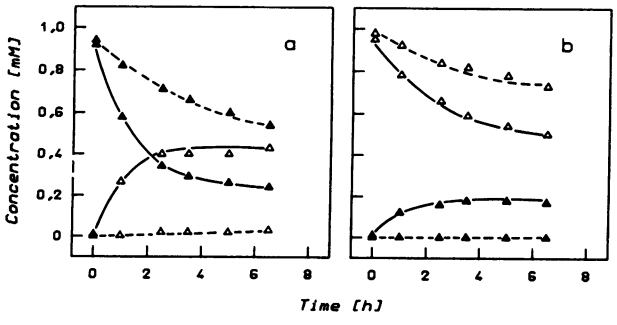

FIG. 2. Isomerization of butyryl-CoA (▲) and isobutyryl-CoA (△) in cell extracts of strain WoG13 and effect of coenzyme B_{12} on the isomerization activity. The assay mixture contained about 3 mg of protein ml^{-1}. (a) Butyryl-CoA as substrate; (b) isobutyryl-CoA as substrate. Experiments were carried out in the absence (– – –) and presence (——) of 20 μM coenzyme B_{12}.

TABLE 1. Enzymes involved in butyrate-to-isobutyrate isomerization by cell extracts of strain WoG13

Enzyme	EC no.	Sp act (nmol/min · mg of protein^{-1})
Butyryl-CoA:isobutyryl-CoA mutase	None	19[a]
		7[b]
Butyryl-CoA:acetate CoA transferase	2.8.3.8	1,700
Isobutyryl-CoA:acetate CoA transferase	2.8.3.8	540

[a] With butyryl-CoA as the substrate.
[b] With isobutyryl-CoA as the substrate.

determined, whereas only 7 nmol/min · mg of protein^{-1} was found with isobutyryl-CoA (Table 1). The isomerase activity depended strictly on the presence of coenzyme B_{12}. In the absence of coenzyme B_{12}, no isobutyryl-CoA or butyryl-CoA was formed (Fig. 2). Other cobalamine derivatives such as vitamin B_{12} or hydroxocobalamine (20 μM each) could not replace adenosyl cobalamine (data not shown). The slight decrease of total CoA ester content during incubation was probably caused by hydrolysis. No isomerization was detected with free butyrate or isobutyrate as the substrate.

A butyryl-CoA:acetate CoA transferase activity (1.7 μmol/min · mg of protein^{-1}) and an isobutyryl-CoA:acetate CoA-transferase activity (0.54 μmol/min · mg of protein^{-1}) were found in cell extracts.

^{13}C-NMR studies. To study the chemistry of the butyrate-isobutyrate isomerization reaction, the conversion of [3-^{13}C]butyrate in a dense cell suspension of strain WoG13 was investigated. The kinetic pattern was the same as that shown in Fig. 1a. The NMR spectrum of a supernatant sample taken after 6 h of incubation clearly showed a ^{13}C signal of [2-^{13}C]isobutyrate (37.8 ppm) in addition to the signal of [3-^{13}C]butyrate (20.2 ppm) (Fig. 3). In a control experiment with no cells added, no indications of [2-^{13}C] isobutyrate formation could be detected.

Isobutyrate degradation by a defined triculture. In dense cell suspensions composed of strain WoG13 (0.54 mg of protein per ml) and the coculture *S. wolfei-M. hungatei* (1.02 mg of protein per ml), isobutyrate was degraded completely to acetate and methane (Fig. 4). Electron recovery was 107%. Butyrate accumulated transiently to a concentration of 3 mM if the ratio of cell protein of strain WoG13 to that of the binary coculture was 5.5:1. No butyrate, acetate, or methane was formed by a coculture of only *S. wolfei* and *M. hungatei* incubated with isobutyrate.

DISCUSSION

In the present study, the isomerization of butyrate to isobutyrate and the reverse are documented for the first time with a pure culture of a strictly anaerobic bacterium, strain WoG13. So far, this conversion was observed under anaerobic conditions only with lake sediment samples or undefined enrichment cultures: butyrate degradation was inhibited by the addition of hydrogen in sediment samples, and isobutyrate accumulated (7). Approximately 50% of the added butyrate was isomerized to isobutyrate. In a methanogenic enrichment culture obtained with isobutyrate, Tholozan and coworkers (14) showed isobutyrate degradation with a transient accumulation of butyrate and also the reverse reaction. A similar pattern of isobutyrate fermentation was observed with a mesophilic enrichment culture (12), and it was suggested that, in natural anoxic environments, isomerization of isobutyrate to butyrate plays an important role as the first step in methanogenic degradation of this branched fatty acid. In the present study, degradation of isobutyrate to acetate and methane by a defined triculture was shown for the first time. In this artificially mixed culture, strain WoG13 isomerized isobutyrate to butyrate, and the latter was oxidized to acetate and methane by *S. wolfei* and

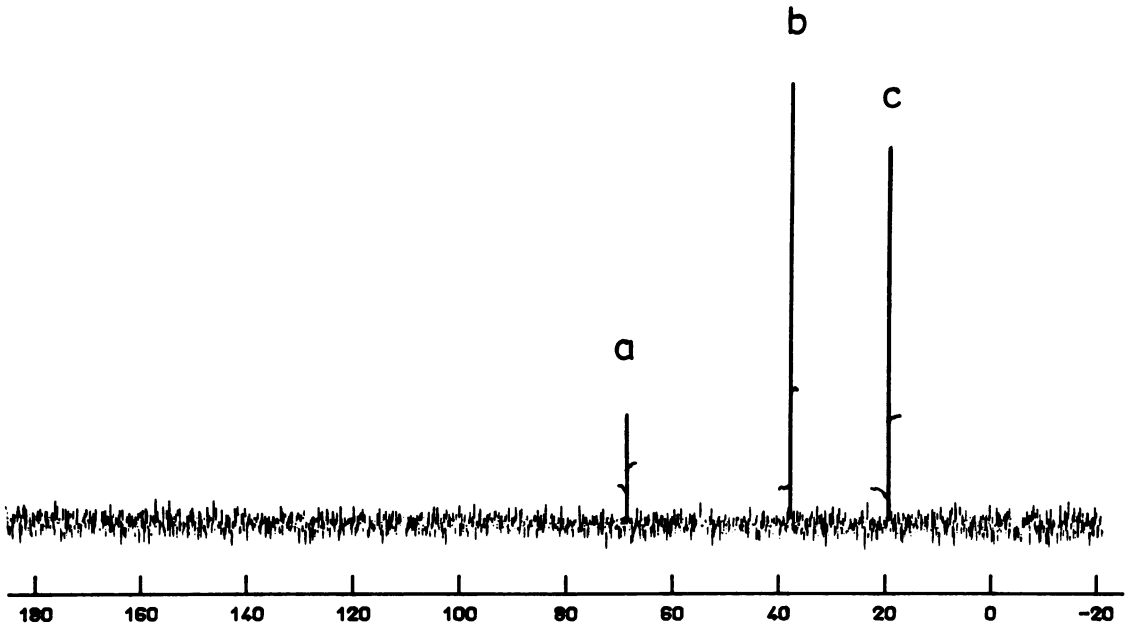

FIG. 3. ^{13}C-NMR spectrum of the supernatant of a dense cell suspension of strain WoG13 incubated with [3-^{13}C]butyrate for 6 h. Dioxane was used as reference for chemical shifts. (a) Dioxane (67.4 ppm); (b) [2-^{13}C]isobutyrate (37.8 ppm); (c) [3-^{13}C]butyrate (20.2 ppm).

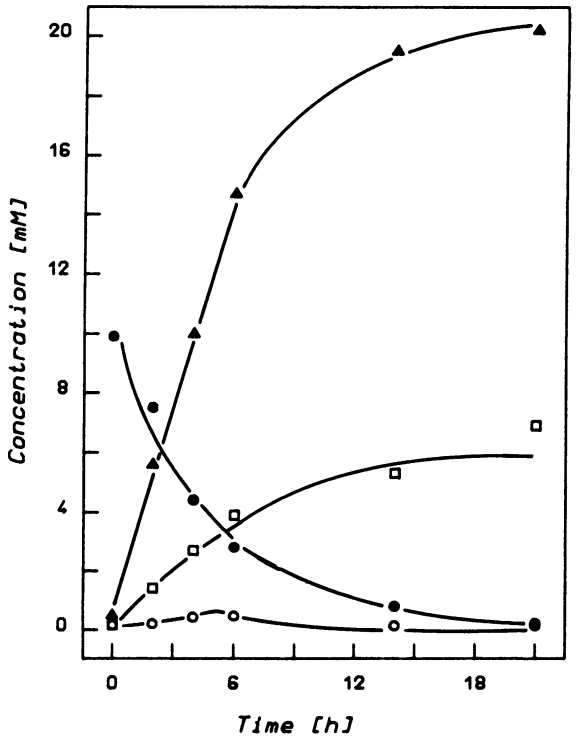

FIG. 4. Isobutyrate degradation by a defined triculture composed of strain WoG13 (0.54 mg of protein ml^{-1}) and the coculture *S. wolfei-M. hungatei* (1.02 mg of protein ml^{-1}). Symbols: ●, isobutyrate; ○, butyrate; ▲, acetate; □, methane.

M. hungatei. This reaction sequence involves the following reaction steps (free energy changes calculated as described previously [4, 13]):

$$\text{Isobutyrate}^- \longrightarrow \text{Butyrate}^- \quad (1)$$
$$\Delta G^{o\prime} \approx 0 \text{ kJ/mol}$$

$$2\ \text{Butyrate}^- + 2H_2O \longrightarrow 4\ \text{Acetate}^- + 4H_2 + 2H^+ \quad (2)$$
$$\Delta G^{o\prime} = +96.2 \text{ kJ/2 mol of butyrate}$$

$$4H_2 + CO_2 \longrightarrow CH_4 + 2H_2O \quad (3)$$
$$\Delta G^{o\prime} = -131.0 \text{ kJ/mol of } CH_4$$

$$\text{Total: } 2\ \text{Isobutyrate}^- + 2H_2O + CO_2 \longrightarrow 4\ \text{Acetate}^- + 2H^+ + CH_4 \quad (4)$$
$$\Delta G^{o\prime} = -34.8 \text{ kJ/2 mol of isobutyrate}$$

Since the first reaction does not release free energy, it has to be catalyzed either cometabolically by an organism which has no advantage from this process or by a bacterium which profits energetically from a later step, e.g., butyrate fermentation to acetate and molecular hydrogen. The former appears to be true for the system described here. However, repeated transfer of an isobutyrate-degrading methanogenic enrichment culture (12) and a preliminary report on a defined methanogenic coculture growing with isobutyrate (17a) indicate that isobutyrate-butyrate isomerization can also be carried out by bacteria which obtain energy in further butyrate degradation.

The reverse reaction, namely, butyrate conversion to isobutyrate, may have an ecological function in the maintenance of a constant isobutyrate pool for reductive valine synthesis (1), especially in an environment poor in protein supply.

Our experiments with cell extracts and ^{13}C-NMR studies prove that the reversible conversion of butyrate to isobutyrate requires activation of these fatty acids to their CoA derivatives and that the rearrangement represents a migration of the carboxyl group to the adjacent carbon atom (Fig. 5). Such rearrangements exchanging the position of an organic moiety and a hydrogen atom on adjacent carbon atoms are known to be catalyzed by mutases, e.g., methylmalonyl-CoA mutase, glutamate mutase, and methylitaconate mutase (2). These enzyme activities typically depend on coenzyme B_{12}, and this is also the case with the butyryl-CoA:isobutyryl-CoA mutase of our strain WoG13. The same was true for the mutase of *S. cinnamonensis* (3). With respect to coenzyme B_{12} dependence and the rearrangement pattern (see above), the two enzyme reactions appear to be similar. In contrast, the mutase of strain WoG13 appears to be involved in catabolism whereas the *S. cinnamonensis* enzyme catalyzes a step in secondary metabolism, i.e., biosynthesis of the antibiotic monensin-A.

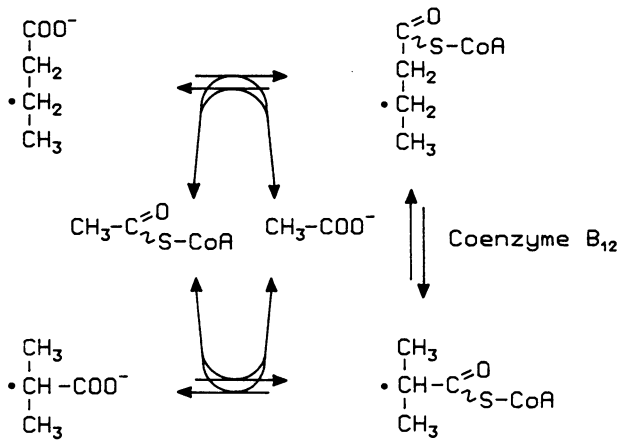

FIG. 5. Scheme of the rearrangement of [3-^{13}C]butyrate to [2-^{13}C]isobutyrate catalyzed by cell suspensions of strain WoG13. The labelled C atom is marked by a dot.

ACKNOWLEDGMENTS

We thank Andreas Brune for helpful instructions in HPLC analysis and J.-L. Tholozan for ^{13}C-NMR analysis.

Financial support from the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

REFERENCES

1. **Allison, M. J., and M. P. Bryant.** 1963. Biosynthesis of branched-chain amino acids from branched-chain fatty acids by rumen bacteria. Arch. Biochem. Biophys. **101:**262–277.
2. **Barker, H. A.** 1972. Coenzyme B_{12}-dependent mutases causing carbon chain rearrangements, p. 509–537. *In* P. D. Boyer (ed.), The enzymes, 3rd ed., vol. 6. Academic Press, Inc., New York.
3. **Brendelberger, G., J. Retey, D. M. Ashworth, K. Reynolds, F. Willenbrock, and J. A. Robinson.** 1988. Die enzymatische Umwandlung von Isobutyryl- zu n-Butyrylcarba(dethia)-Coenzym A: Eine coenzym-B_{12}-abhängige Gerüstumlagerung. Angew. Chemie **100:**1122–1124.
4. **D'Ans, J., and E. Lax.** 1983. Organische Verbindungen, p. 1003–1006. *In* Taschenbuch für Chemiker und Physiker, 4th ed., vol. 2. Springer-Verlag KG, Berlin.
5. **Gani, D., D. O'Hagen, K. Reynolds, and J. A. Robinson.** 1985. Biosynthesis of the polyether antibiotic monensin-A: stereochemical aspects of the incorporation and metabolism of isobutyrate. J. Chem. Soc. Chem. Commun. **372:**1002–1004.
6. **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.

7. **Lovley, D. R., and M. J. Klug.** 1982. Intermediary metabolism of organic matter in the sediments of a eutrophic lake. Appl. Environ. Microbiol. **43:**552–560.
8. **Matthies, C., and B. Schink.** 1992. Fermentative degradation of glutarate via decarboxylation by newly isolated strictly anaerobic bacteria. Arch. Microbiol. **157:**290–296.
9. **Platen, H., and B. Schink.** 1989. Anaerobic degradation of acetone and higher ketones via carboxylation by newly isolated denitrifying bacteria. J. Gen. Microbiol. **135:**883–891.
10. **Reynolds, K., G. Gani, and J. A. Robinson.** 1986. Biosynthesis of monensin. Evidence for a vicinal interchange rearrangement linking n-butyryl-CoA and isobutyryl-CoA. J. Chem. Soc. Chem. Commun. **660:**1334–1336.
11. **Reynolds, K., and J. A. Robinson.** 1985. Biosynthesis of monensin. The intramolecular rearrangement of isobutyryl-CoA to n-butyryl-CoA. J. Chem. Soc. Chem. Commun. **1320:**1831–1832.
12. **Stieb, M., and B. Schink.** 1989. Anaerobic degradation of isobutyrate by methanogenic enrichment cultures and by a *Desulfococcus multivorans* strain. Arch. Microbiol. **151:**126–132.
13. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation of chemotrophic anaerobic bacteria. Bacteriol. Rev. **41:**100–180.
14. **Tholozan, J. L., E. Samain, and J. P. Grivet.** 1988. Isomerization between n-butyrate and isobutyrate in enrichment cultures. FEMS Microbiol. Ecol. **53:**187–191.
15. **Tholozan, J. L., E. Samain, J. P. Grivet, R. Moletta, and H. C. Dubourguier.** 1988. Reductive carboxylation of propionate into butyrate in methanogenic ecosystems. Appl. Environ. Microbiol. **54:**441–445.
16. **Widdel, F., G. W. Kohring, and F. Mayer.** 1983. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. III. Characterization of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov., Arch. Microbiol. **134:**286–294.
17. **Widdel, F., and N. Pfennig.** 1981. Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. Arch. Microbiol. **129:**395–400.

17a. **Wu, W.-M., and M. K. Jain.** 1990. Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, I 50, p. 206.

18. **Zamenhoff, S.** 1957. Preparation and assay of desoxyribonucleic acid from animals tissue. Methods Enzymol. **3:**696–704.