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Anaerobic Degradation of Normal- and Branched-Chain Fatty Acids with Four or More Carbons to Methane by a Syntrophic Methanogenic Triculture

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Syntrophic degradation of normal- and branched-chain fatty acids with 4 to 9 carbons was investigated with a mesophilic syntrophic isobutyrate-butyrate-degrading triculture consisting of the non-spore-forming, syntrophic, fatty acid-degrading, gram-positive rod-shaped strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18. This triculture converted butyrate and isobutyrate to methane and converted valerate and 2-methylbutyrate to propionate and methane. This triculture also degraded caproate, 4-methylvalerate, heptanoate, 2-methylhexanoate, caprylate, and pelargoate. During the syntrophic conversion of isobutyrate and butyrate, a reversible isomerization between butyrate and isobutyrate occurred; isobutyrate and butyrate were isomerized to the other isomeric form to reach nearly equal concentrations and then their concentrations decreased at the same rates. Butyrate was an intermediate of syntrophic isobutyrate degradation. When butyrate was degraded in the presence of propionate, 2-methylbutyrate was synthesized from propionate and isobutyrate formed from butyrate. During the syntrophic degradation of valerate, isobutyrate, butyrate, and 2-methylbutyrate were formed and then degraded. During syntrophic degradation of 2-methylbutyrate, isobutyrate and butyrate were formed and then degraded.

Branched-chain fatty acids, such as isobutyrate and 2-methylbutyrate, are intermediates of the anaerobic digestion of proteins (9). Both have also been detected during simultaneous degradation of acetate, propionate, and butyrate by methanogenic granules (19, 22). These branched-chain fatty acids can be converted either to acetate or completely to CO_2 by some sulfate-reducing bacteria via sulfate reduction (14, 16, 17). 2-Methylbutyrate can be converted to acetate, propionate, and methane by syntrophic butyrate degraders such as *Syntrophospora bryantii* (13) and strains NSF-2 and SF-1 (12), together with H_2-utilizing methanogens. The syntrophic conversion of isobutyrate in the absence of sulfate has been studied with digested sludges (1, 15, 25). A reversible isomerization between butyrate and isobutyrate indicates that butyrate is an intermediate of anaerobic isobutyrate degradation (15). The appearance of isobutyrate during syntrophic butyrate degradation is due to the isomerization performed by some organisms involved in isobutyrate degradation. The mechanism of formation of 2-methylbutyrate is unknown.

To date, only one syntrophic butyrate-degrading culture, the straight rod-shaped *Syntrophospora*-like strain SF-1, has been reported to use isobutyrate (12). However, the utilization of isobutyrate by this strain was questioned (6, 14). We isolated a defined triculture that would syntrophically degrade butyrate and isobutyrate, perform reciprocal isomerization between butyrate and isobutyrate, and convert acetate and H_2 (or formate) to methane (4, 21). Recently, an anaerobic bacterium, strain WoG13, which grows on dicarboxylic acid glutarate and which also performs reciprocal isomerization of butyrate and isobutyrate has been isolated (8). Isobutyrate can be degraded by a triculture consisting of strain WoG13, *Syntrophomonas wolfei*, and *Methanospirillum hungatei*. In this triculture, strain WoG13 isomerized isobutyrate to butyrate and *S. wolfei* together with *Methanospirillum hungatei* converted butyrate to acetate and methane.

Our triculture, consisting of a syntrophic isobutyrate-butyrate degrading rod (strain IB) along with *Methanobacterium formicicum* T1N and *Methanosarcina mazei* T18, was isolated from syntrophic methanogenic granules (21). This triculture can also utilize 2-methylbutyrate, valerate, and other branched- and normal-chain fatty acids, with up to nine carbon atoms, as substrates and can synthesize 2-methylbutyrate during syntrophic butyrate degradation in the presence of propionate. In the present communication, we describe the isomerization and syntrophic conversion of isobutyrate, butyrate, and other normal- and branched-chain fatty acids by this defined triculture.

(Preliminary results of this study have been presented at annual meetings of the American Society for Microbiology [4, 20].)

MATERIALS AND METHODS

Chemicals and gases. All chemicals used were of analytical grade and were obtained from Sigma Chemical Co., St. Louis, Mo., or Mallinckrodt Inc., Paris, Ky. A gas mixture of N_2-CO_2 (70:30) was obtained from Union Carbide Corp. (Linde Div., Warren, Mich.) and passed over heated (350°C) copper filings to remove traces of O_2.

Sources of cultures. The syntrophic isobutyrate-butyrate-degrading strain IB was isolated by us earlier (21). It was isolated from syntrophic methanogenic granules developed on a volatile fatty acid mixture consisting of acetate, propionate, and butyrate, along with *Methanobacterium formicicum* T1N

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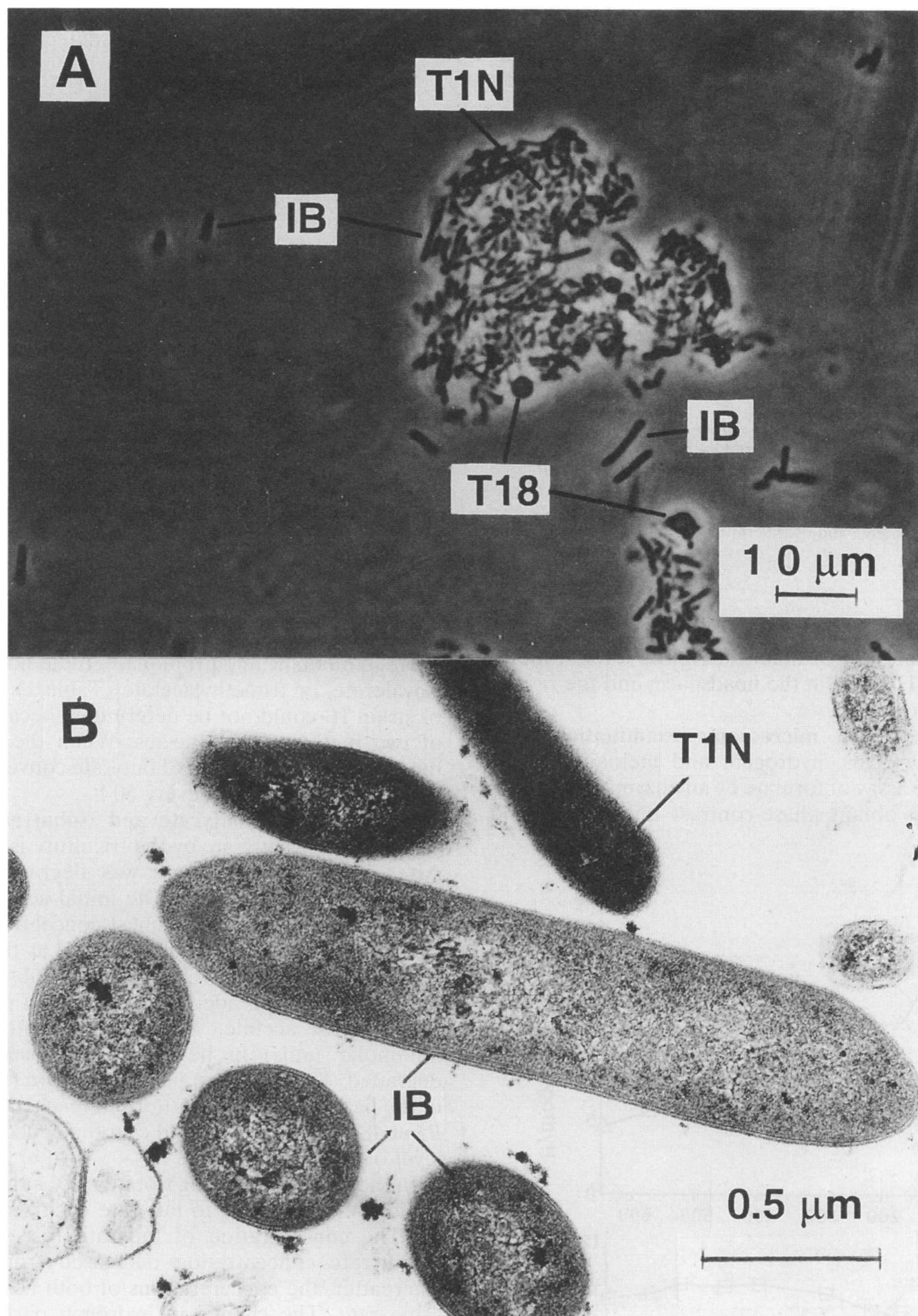

FIG. 1. (A) Phase-contrast microphotograph of triculture. IB, syntrophic isobutyrate-butyrate degrader strain IB; T1N, *Methanobacterium formicicum* T1N; T18, *Methanosarcina mazei* T18. (B) Transmission electron microphotograph of a thin section of a cell of strain IB.

(DSM 6298) and *Methanosarcina mazei* T18 (DSM 6230) as syntrophic partners.

Medium and experimental procedures. Liquid media and solutions were prepared and sterilized under strictly anaerobic conditions (23). The experiments were carried out in 158-ml serum vials (Wheaton Scientific, Millville, N.J.) containing 50 ml of liquid medium. The phosphate-buffered basal medium (5) used for the growth of the syntrophic triculture containing strain IB was supplemented with the following (per 100 ml): vitamin solution (18), 1.0 ml; 2.5% $Na_2S \cdot 9H_2O$ (wt/vol), 0.6 ml; 10% $NaHCO_3$ (wt/vol), 4.0 ml; and a K_2HPO_4-KH_2PO_4 mixture (29%:15%, wt/vol), 0.6 ml. Substrate was supplied by adding stock solutions of sodium salt of fatty acids to the medium to obtain initial concentrations of 6 to 10 mM (each) for butyrate, isobutyrate, valerate, 2-methylbutyrate, caproate, and 4-methylbutyrate, 5 mM (each) for heptanoate and 2-methylhexanoate, and 2.5 mM (each) for caprylate and pelargoate. The vials were pressurized with N_2-CO_2 mixture (70:30) to 72 kPa (0.71 atm), resulting in a pH of about 7.0 in the media.

All the experiments were carried out at 37°C with a shaking incubator. A well-grown syntrophic triculture containing strain

TABLE 1. End products of volatile fatty acid degradation by a defined isobutyrate-butyrate-degrading culture[a]

Substrate[b]		Product concn (mmol/mmol of substrate consumed)	
Acid	No. of carbons	CH_4	Propionate
Butyrate	4	2.46	0
Isobutyrate	4	2.44	0
Valerate	5	1.52	0.91
2-Methylbutyrate	5	1.49	0.92
Caproate	6	3.84	0
4-Methylvalerate	6	3.82	0
Heptanoate	7	2.46	0.99
2-Methylhexanoate	7	2.49	0.97
Caprylate	8	4.65	0
Pelargoate	9	3.72	0.90

[a] The experiment was performed at 35°C for 3 months. The initial volatile fatty acid concentrations were 10 mM for the acids with four to six carbon atoms, 5 mM for those with seven atoms, and 2.5 mM for those with eight to nine atoms. The initial pH was 7.0 to 7.1.

[b] Propionate, trimethylacetate, and isovalerate were not degraded by the triculture.

IB was used as the inoculum (4 to 20%). The fatty acid degradation was monitored by determining the level of methane production, the H_2 level in the headspace, and the rate of substrate consumption.

Analytical methods and microscopic examination. The quantitation of fatty acids, hydrogen, and methane by gas chromatography, the assay of formate by an enzymatic method, and the methods to obtain phase-contrast microscopic and transmission electron microphotographs have been described previously (19). The minimum detectable concentration of formate was 5 μM.

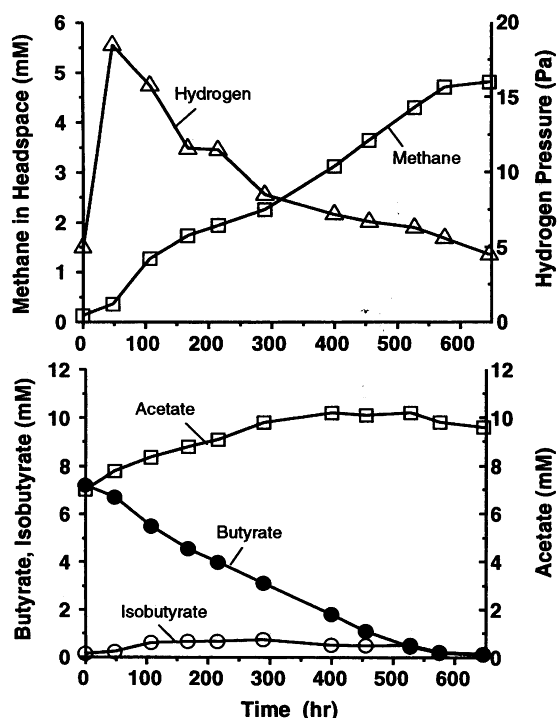

FIG. 2. Degradation of butyrate by the triculture consisting of strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18. Acetate and methane were end products of syntrophic butyrate degradation. Hydrogen and isobutyrate were observed during butyrate degradation.

RESULTS

Morphology and substrate utilization. The syntrophic isobutyrate-butyrate-degrading triculture consisted of three distinguishable bacterial strains. Cells of strain IB were nonfluorescent under UV light, slender, and cylindrical, with dimensions of 0.45 by 2.0 to 3.5 μm. Slender or cylindrical fluorescent rods with dimensions of 0.35 by 1.2 to 3.0 μm were identified as *Methanobacterium formicicum* T1N, while the coccus- and sarcina-type cells were identified as *Methanosarcina mazei* T18 (21). The cells of strain IB and strain T1N grew homogeneously to form clumps with strain T18 (Fig. 1A). The TEM examination revealed that the cell walls of strain IB cells have a typical gram-positive structure (Fig. 1B). Neither spore nor poly-β-hydroxybutyrate structures were observed. This is different from *Syntrophospora* species, which form spores (13, 14), and *Syntrophomonas* species, which contain poly-β-hydroxybutyrate (2, 3, 8).

This triculture converted isobutyrate, butyrate, caproate, 4-methylvalerate, and caprylate completely to methane and converted valerate, 2-methylbutyrate, heptanoate, and pelargoate to methane and propionate. It did not utilize propionate, isovalerate, or trimethylacetate (Table 1). The doubling time of strain IB could not be determined because of the presence of two methanogenic species. When the triculture grew on butyrate at 37°C, the rate of butyrate conversion to acetate was doubled approximately every 60 h.

Degradation of butyrate and isobutyrate. Results of the degradation of butyrate by the triculture is presented in Fig. 2. After inoculation, butyrate was degraded with concurrent production of isobutyrate. The initial acetate was a carryover from the inoculum. The highest concentration of isobutyrate detected was 0.75 mM. This occurred as the butyrate concentration decreased from 7.2 to 3.1 mM. Subsequently, both were simultaneously degraded with concurrent production and consumption of acetate. Methane concentration (reported in millimolar units) in headspace increased as butyrate was degraded. The hydrogen partial pressure (reported in pascals) in the headspace of the serum vial reached a peak level of 18.5 Pa at 48 h of incubation and eventually decreased to the initial level of 4.8 Pa.

In the experiment using isobutyrate as the substrate, isomerization of isobutyrate to butyrate occurred immediately (Fig. 3). The concentration of butyrate rose to 2.4 mM as the isobutyrate concentration decreased from 8.0 to 2.7 mM. Thereafter, the concentrations of both acids decreased at the same rate. The change in hydrogen partial pressure in the headspace and the change in rate of acetate production and consumption were similar to the time course profile observed for butyrate degradation. Methane concentration in the headspace increased as isobutyrate was degraded. The highest H_2 partial pressure observed was 23 Pa.

When the triculture was inoculated in the medium containing almost equal concentrations of butyrate and isobutyrate (4.4 and 4.1 mM, respectively), the concentrations of the two acids decreased at approximately the same rate until both were completely consumed (Fig. 4). The methane concentration in the headspace increased as both butyrate and isobutyrate were degraded. The peak H_2 partial pressure observed during this experiment was 16 Pa. Formate was not at detectable level in these or any of the previous experiments.

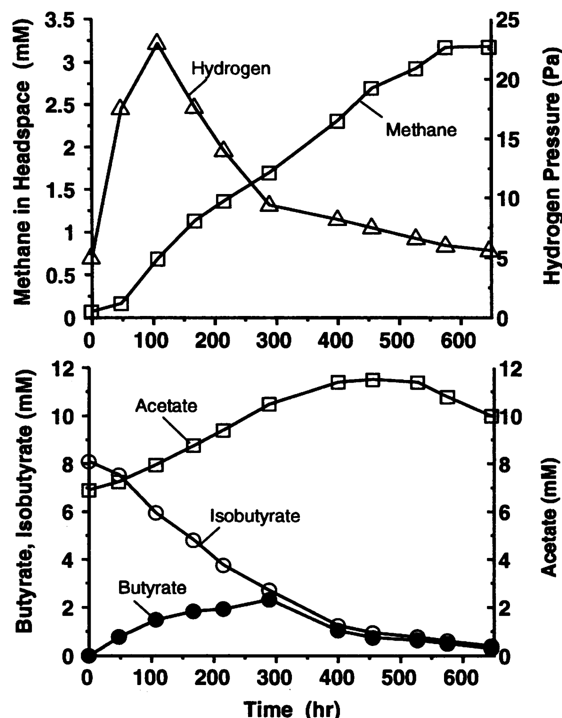

FIG. 3. Degradation of isobutyrate by the triculture consisting of strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18. Acetate and methane were end products of syntrophic isobutyrate degradation. Hydrogen and butyrate were observed during isobutyrate oxidation.

These results demonstrate that strain IB carries out a reversible isomerization between isobutyrate and butyrate during syntrophic oxidation of butyrate or isobutyrate, since neither *Methanobacterium formicicum* nor *Methanosarcina mazei* utilizes butyrate or isobutyrate.

Degradation of valerate and 2-methylbutyrate. During the time course for degradation of valerate (initial concentration of 7.4 mM), the propionate concentration increased to 6.8 mM (Fig. 5). The molar ratio between propionate produced and valerate consumed was 0.92. During the experiment, isobutyrate, butyrate, and 2-methylbutyrate were also produced. Isobutyrate, butyrate, and 2-methylbutyrate appeared almost simultaneously after 45 h of growth. The concentration of butyrate formed was slightly higher than the concentration of isobutyrate formed. Butyrate and isobutyrate disappeared with the consumption of valerate prior to the disappearance of 2-methylbutyrate. The methane concentration in the headspace increased as valerate was degraded. The highest hydrogen partial pressure (5.6 Pa) observed during valerate conversion was much lower than that observed during butyrate or isobutyrate conversion.

In another experiment 2-methylbutyrate was used as the substrate at 6.7 mM (Fig. 6). Propionate was produced as 2-methylbutyrate was converted at a molar ratio of 0.91 (propionate formed/2-methylbutyrate consumed). In addition to propionate, isobutyrate was also produced, and its concentration reached a maximum of 0.27 mM after 116 h. Butyrate, which was observed only after isobutyrate was produced, reached a maximum level (0.06 mM) after 168 h. Isobutyrate was probably isomerized to butyrate rather than butyrate being produced directly. Both butyrate and isobutyrate were consumed prior to the complete consumption of 2-methylbutyrate. No detectable level of valerate was observed during the experiment. The methane concentration in the headspace increased as 2-methylbutyrate was degraded. The maximum hydrogen partial pressure (5.3 Pa) detected was similar to that detected during valerate degradation. Formate was not detected in the triculture containing either valerate or 2-methylbutyrate during their degradation.

Syntrophic butyrate degradation in the presence of propionate. In this experiment, butyrate and propionate were added to a well-grown triculture to achieve starting concentrations of 6.0 and 6.1 mM, respectively. As is shown in Fig. 7, the isomerization of butyrate to isobutyrate occurred immediately. A significant amount of 2-methylbutyrate was also produced. This appeared to be coupled in the initial hours of growth with the disappearance of propionate. After 55 h, the butyrate concentration decreased to a low level (0.1 mM). The highest concentration of 2-methylbutyrate accumulated, 0.91 mM, corresponded to a decrease in propionate concentration from 6.1 mM to 5.2 mM. When 2-methylbutyrate was consumed, the concentration of propionate rose to its original level. The methane concentration in the headspace increased as butyrate was degraded. The trend of the change in hydrogen partial pressure in the headspace was similar to trends in other experiments, with the highest concentration being 9 Pa. The acetate concentration in the culture increased from an initial 14.9 mM to 24.9 mM during the assay because of the slow conversion of acetate to methane.

To test if propionate degradation was carried out by strain IB and if 2-methylbutyrate could be formed in the absence of isobutyrate or butyrate, a control experiment was performed by inoculating the triculture into a medium containing 10 mM acetate and 10 mM propionate. No propionate was degraded

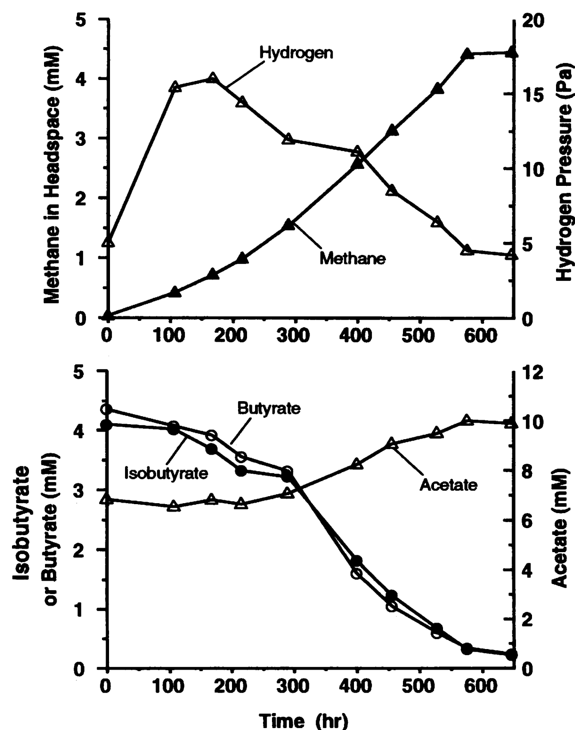

FIG. 4. Degradation of equal molar concentrations of isobutyrate and butyrate by the triculture consisting of strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18.

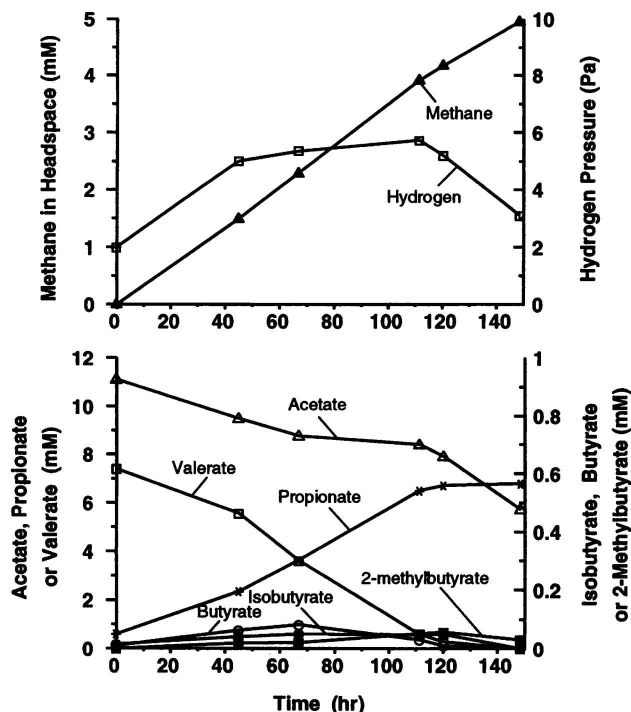

FIG. 5. Degradation of valerate by the triculture consisting of strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18. Acetate, propionate, and methane were end products of syntrophic valerate degradation. Butyrate, isobutyrate, and 2-methylbutyrate were observed during valerate degradation.

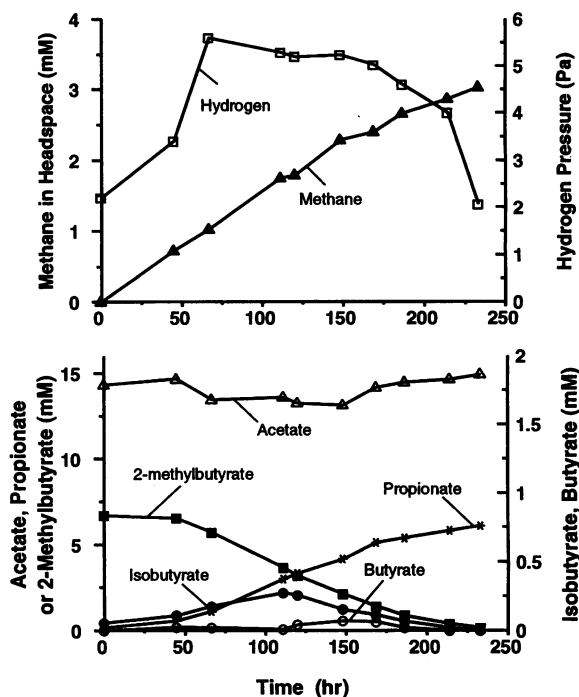

FIG. 6. Degradation of 2-methylbutyrate by the triculture consisting of strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18. Acetate, propionate, and methane were end products of 2-methylbutyrate degradation. Butyrate and isobutyrate were observed during the degradation.

and no other fatty acids were formed even after 3 months of incubation. However, acetate was degraded and methane was produced. These results demonstrated that 2-methylbutyrate was synthesized from propionate only when butyrate and isobutyrate were present.

DISCUSSION

A triculture, consisting of the syntrophic fatty acid-degrading rod strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18, was able to degrade fatty acids to methane. Strain IB is the first isolated syntrophic isobutyrate and butyrate degrader which can isomerize isobutyrate to butyrate and then degrade it. One remarkable characteristic of syntrophic isobutyrate or butyrate degradation by the triculture was that nearly equal concentrations of isobutyrate and butyrate were eventually achieved and then the concentrations of both the fatty acids decreased at the same rates. This was not observed during the isobutyrate or butyrate degradation in anaerobically digested sludges or methanogenic granules (1, 15, 22) or by the triculture consisting of strain WoG13, *S. wolfei*, and *Methanospirillum hungatei* (8). In those cases, butyrate always disappeared more rapidly than isobutyrate because syntrophic butyrate degraders, such as *Syntrophospora* species (13, 24) and *Syntrophomonas* species (10, 11), that use butyrate but do not utilize isobutyrate were present. Another characteristic was that the isomerization between butyrate and isobutyrate is coupled to syntrophic butyrate oxidation and therefore can be completely inhibited in the presence of high hydrogen partial pressure or high formate concentration (20). This is different from the isomerization between butyrate and isobutyrate performed in a lake sediments (7) and by strain WoG13 (8). In those cases, the isomerization was independent of syntrophic butyrate degradation. However, the physiological reason for isomerization of butyrate to isobutyrate is not clear, since the isobutyrate formed has to be reisomerized to butyrate for further degradation. More research is needed to understand this phenomenon.

The syntrophic degradation of 2-methylbutyrate and valerate appeared to be more complicated than degradation of the acids with four carbon atoms. Although these two fatty acids, like butyrate and isobutyrate, have similarity in their molecular structures, a reversible isomerization between 2-methylbutyrate and valerate has not been observed. In our experiments, isomerization from 2-methylbutyrate to valerate did not occur, because no valerate was detected during 2-methylbutyrate conversion, but the isomerization from valerate to 2-methylbutyrate indeed did exist, because a trace amount of 2-methylbutyrate was repeatedly detected during the syntrophic conversion of valerate.

During the syntrophic conversion of 2-methylbutyrate, propionate was formed at a molar ratio of 0.91 (propionate produced/2-methylbutyrate consumed), but isobutyrate and butyrate also occurred one after another. The results of butyrate conversion in the presence of propionate provided information useful for understanding the mechanism of the formation of 2-methylbutyrate and for examining the syntrophic conversion of 2-methylbutyrate. During butyrate degradation in the presence of propionate, 2-methylbutyrate was formed in addition to isobutyrate, which was formed as an isomerized product. The synthesis of 2-methylbutyrate was related to the presence of propionate and isobutyrate, because (i) the increment of 2-methylbutyrate was proportional to the decrement of propionate, and the propionate concentration

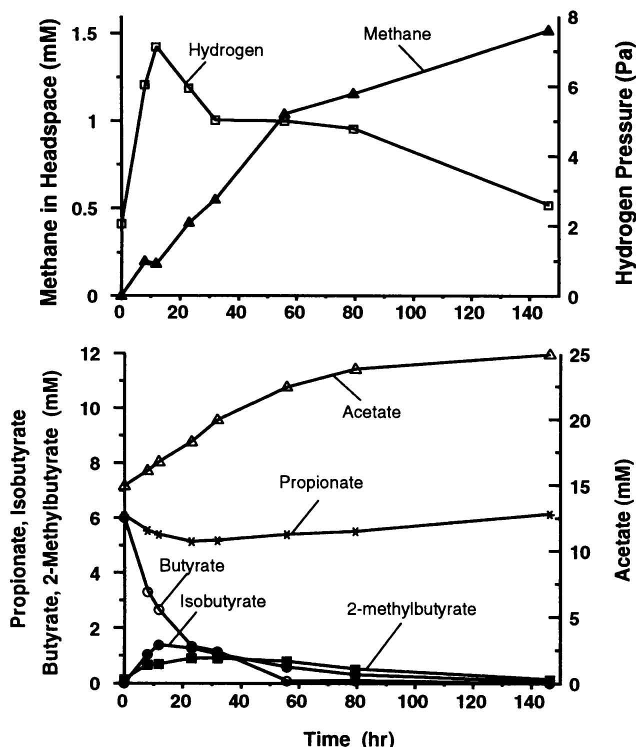

FIG. 7. Degradation of butyrate in the presence of propionate by the triculture consisting of strain IB, *Methanobacterium formicicum* T1N, and *Methanosarcina mazei* T18. Acetate and methane were end products of butyrate degradation. Isobutyrate and 2-methylbutyrate were observed during the degradation.

was restored to its original level when the 2-methylbutyrate formed was consumed (Fig. 7); (ii) no 2-methylbutyrate was formed in the presence of acetate plus propionate without isobutyrate or butyrate; and (iii) isobutyrate was detected prior to the detection of butyrate during 2-methylbutyrate conversion (Fig. 6). However, further research is needed to detail the pathway of the synthesis of 2-methylbutyrate.

When the triculture was grown on valerate, propionate was produced (Fig. 5) at a molar ratio of 0.92 (propionate produced/valerate converted). The syntrophic conversion of valerate to methane and propionate probably followed β-oxidation, as has been shown to be the case in other syntrophic butyrate-degrading cultures (10, 13). However, the formation of isobutyrate, butyrate, and 2-methylbutyrate during valerate conversion indicates that other pathways may also exist. The formation of 2-methylbutyrate may be due to the reversible isomerization between valerate and 2-methylbutyrate, since butyrate was isomerized to isobutyrate during butyrate degradation. The pathway of formation of butyrate during valerate degradation is still not clear.

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REFERENCES

1. **Aguilar, A., C. Casas, J. Lafuente, and J. M. Lema.** 1990. Kinetic modelling of isomerization and anaerobic degradation of n- and i-butyrate. J. Ferment. Bioeng. **69:**261–264.
2. **Amos, D. A., and M. J. McInerney.** 1989. Poly-β-hydroxyalkanoate in *Syntrophomonas wolfei*. Arch. Microbiol. **152:**172–177.
3. **Dubourguier, H. C., D. B. Archer, G. Albagnac, and G. Prensier.** 1988. Structure and metabolism of methanogenic microbial conglomerates, p. 13–25. *In* E. R. Hall and P. N. Hobson (ed.), Anaerobic digestion 1988. Pregramon Press, Oxford, United Kingdom.
4. **Jain, M. K., W. Wu, and J. G. Zeikus.** 1989. Isomerization mediated conversion of iso-butyrate and butyrate to methane by syntrophic biomethanation consortia, p. 313. Abstr. 89th Annu. Meet. Am. Soc. Microbiol. 1989. American Society for Microbiology, Washington, D.C.
5. **Kenealy, W., and J. G. Zeikus.** 1981. Influence of corrinoid antagonists on methanogen metabolism. J. Bacteriol. **146:**133–140.
6. **Lorowitz, W. H., H. Zhao, and M. P. Bryant.** 1989. *Syntrophomonas wolfei* subsp. *saponavida* subsp. nov., a long-chain fatty-acid-degrading, anaerobic, syntrophic bacterium; *Syntrophomonas wolfei* subsp. *wolfei* subsp. nov.; and emended descriptions of the genus and species. Int. J. Syst. Bacteriol. **39:**122–126.
7. **Lovely, 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. Reciprocal isomerization of butyrate and isobutyrate by the strictly anaerobic bacterium WoG13 and methanogenic isobutyrate degradation by a defined triculture. Appl. Environ. Microbiol. **58:**1435–1439.
9. **McInerney, M. J.** 1988. Anaerobic hydrolysis and fermentation of fats and proteins, p. 373–416. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.
10. **McInerney, M. J., M. P. Bryant, and J. W. Costerton.** 1981. *Syntrophomonas wolfei* gen. nov., an anaerobic, syntrophic, fatty acid-oxidizing bacterium. Appl. Environ. Microbiol. **41:**1029–1039.
11. **Roy, F., E. Samain, H. C. Dubourguier, and G. Albagnac.** 1986. *Syntrophomonas sapovorans* sp. nov., a new obligately proton reducing anaerobe oxidizing saturated and unsaturated long chain fatty acids. Arch. Microbiol. **145:**142–147.
12. **Shelton, D. R., and J. M. Tiedje.** 1984. Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. **48:**840–848.
13. **Stieb, M., and B. Schink.** 1985. Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium. Arch. Microbiol. **140:**387–390.
14. **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.
15. **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.
16. **Widdel, F.** 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469–586. *In* A. J. B. Zehnder (ed.), Biology of anaerobic microorganisms. John Wiley & Sons, New York.
17. **Widdel, F., and N. Pfennig.** 1984. Dissimulatory sulfate- or sulfur-reducing bacteria, p. 663–679. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. Williams & Wilkins, Baltimore.
18. **Wolin, E. A., M. R. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238:**2882–2886.
19. **Wu, W.-M., R. F. Hickey, and J. G. Zeikus.** 1991. Characterization of metabolic performance of methanogenic granules treating brewery wastewater: role of sulfate-reducing bacteria. Appl. Environ. Microbiol. **57:**3438–3449.
20. **Wu, W.-M., and M. K. Jain.** 1990. Mechanism of isomerization and interspecies electron transfer during isobutyrate-butyrate degradation by a syntrophic biomethanation triculture, p. 206. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990. American Society for Microbiology, Washington, D.C.
21. **Wu, W.-M., M. K. Jain, E. Conway de Macario, J. H. Thiele, and J. G. Zeikus.** 1992. Microbial composition and characterization of prevalent methanogens and acetogens isolated from syntrophic methanogenic granules. Appl. Microbiol. Biotechnol. **38:**282–290.
22. **Wu, W.-M., J. H. Thiele, M. K. Jain, and J. G. Zeikus.** 1993. Metabolic properties and kinetics of methanogenic granules.

Appl. Microbiol. Biotechnol. **39**:804–811.

23. **Zeikus, J. G., A. Ben-Bassat, and P. W. Hegge.** 1980. Microbiology of methanogenesis in thermal, volcanic environments. J. Bacteriol. **143**:432–464.
24. **Zhao, H., D. Yang, C. R. Woese, and M. P. Bryant.** 1990. Assignment of *Clostridium bryantii* gen. nov., comb. nov., on the basis of a 16S rRNA sequence analysis of its crotonate-grown pure culture. Int. J. Syst. Bacteriol. **40**:40–44.
25. **Zinder, S. H., S. C. Cardwell, T. Anguish, M. Lee, and M. Koch.** 1984. Methanogenesis in a thermophilic (58°C) anaerobic digester: *Methanothrix* sp. as an important aceticlastic methanogen. Appl. Environ. Microbiol. **47**:796–807.