Anaerobic degradation of long-chain dicarboxylic acids by methanogenic enrichment cultures

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Abstract: Methanogenic enrichment cultures fermented the long-chain dicarboxylates adipate, pimelate, suberate, azelate, and sebacate (C_6–C_{10}) stoichiometrically to acetate and methane. After several transfers, the cultures contained cells of only a few morphologically distinguishable types. During anaerobic degradation of dicarboxylic acids with even-numbered carbon atoms, propionate accumulated intermediately, and butyrate was the intermediate product of degradation of those with an odd number of carbon atoms. Degradation of the long-chain dicarboxylates depended strictly on the presence of hydrogenotrophic methanogens. The primary attack in these processes was β-oxidation rather than decarboxylation. A general scheme of anaerobic degradation of long-chain dicarboxylic acids has been deduced from these results.

Key words: Dicarboxylic acid; Anaerobic degradation; Syntrophic oxidation

Introduction

In nature, long-chain dicarboxylic acids are not very abundant. Glutarate and adipate accumulate in sugar beets. Dicarboxylic acids are formed (i) as products of biterminal *n*-alkane oxidation by several *Candida* species [1], and (ii) as intermediate metabolites during aerobic degradation of long-chain alkyl diamines [2] or cyclic alcohols [3] by bacteria.

Fermentative degradation of dicarboxylic acids can follow basically two different reaction schemes: either the substrate is decarboxylated to a fatty acid which undergoes subsequent β-oxidation, or the dicarboxylate is first β-oxidized and decarboxylated afterwards. Direct decarboxylation has been observed only with dicarboxylates of 2–5 C-atoms. Fermenting bacteria have been isolated which conserve the decarboxylation energy (approx. -25 kJ mol^{-1}) as sole energy source for growth [4–7], mostly after the respective substrate has been modified to activate the α-C-atom for decarboxylation.

Anaerobic degradation of dicarboxylates with more than 5 C-atoms has so far only been reported to occur in the presence of NO_3^-, or SO_4^{2-} as electron acceptor [8,9]. The present study doc-

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uments fermentative degradation of such dicarboxylates, and provides evidence of β-oxidation to be the primary step in these degradation processes.

Materials and Methods

Source of microorganisms and mud samples for enrichment cultures

Enrichment cultures were inoculated with the following anoxic mud samples: (1) KoAd with domestic anoxic sewage sludge; (2) WoAd and WoPim with anoxic sediment of a polluted creek near Konstanz, FRG; (3) MaPim, MaAd with anoxic marine black mud of Rio Marin, Venice, Italy; (4) CuPim, CuAd with anoxic black mud from the North Sea coast near Cuxhaven, FRG.

Desulfovibrio vulgaris strain Marburg and *Methanospirillum hungatei* strain SK were kindly provided by Prof. R.K. Thauer, Marburg, and Prof. F. Widdel, Bremen (both FRG).

Media, cultivation and isolation

Enrichment cultures were grown in carbonate-buffered, sulphide-reduced mineral salt medium [10,11]. All procedures of cultivation and isolation were described earlier [10,11]. Growth was followed by measuring optical density at 450 nm.

Experiments with dense cell suspensions were carried out in 25-ml serum bottles filled half with washed cells resuspended in fresh medium (OD_{450} = 0.2–0.6), under N_2/CO_2 (90%/10%), and sealed with butyl rubber stoppers. The protein content of cell suspensions was estimated through an experimentally derived conversion factor (OD_{450} = 0.1 corresponds to 25 mg dry cell matter per liter or 12.5 mg protein per liter).

Growth and cell suspension studies were repeated at least two times; the figures are representative of typical cultures.

Chemical analyses

Fatty acids and methane were measured by gas chromatography as described earlier [7,12]. Dicarboxylic acids were quantified with a Beckman System Gold high pressure liquid chromatograph. A column (4.6 × 45 mm) filled with Ultrasphere 5 mm Spherical 80 Å Pore (C-18) stationary phase (Beckman, Munich, FRG) was used. Elution of samples (20 μl) was carried out isocratically with a potassium phosphate buffer (10 mM, pH 2.4)/methanol solvent system (% (v/v) methanol: 15% for adipate, 20% for pimelate, 30% for suberate, and 45% for azelate and sebacate) at a flow rate of 1 ml min^{-1}. The dicarboxylic acids were detected in a Beckman 167 or 166 detector at 206 nm.

Chemicals

All chemicals used were of reagent grade quality and obtained from Fluka, Neu-Ulm, and

Fig. 1. Microphotographs of cells of the enrichment cultures WoAd (a), WoPim (b) and MaPim (c). Bars equal 10 μm.

Merck, Darmstadt, FRG. All gases were obtained from Messer-Griesheim, Darmstadt, FRG.

Results

Description of methanogenic enrichment cultures

Enrichment cultures with 10 mM adipate (C_6) or pimelate (C_7) in either freshwater or saltwater medium were inoculated with anoxic mud from various freshwater and saltwater sources. After 1–3 weeks of incubation, turbidity increased and gas production started. In further subcultures (3–5 transfers), stable mixed cultures developed. The bacterial populations in the enrichment cultures KoAd and WoAd with adipate looked nearly identical in phase contrast microscopy. As shown in Fig. 1a, short, rod-shaped cells occurring mostly in pairs, and irregular, flattened cocci dominated. In the enrichment culture WoPim (Fig. 1b) three cell types prevailed: slightly curved rods, motile vibrioid rods, and long, fluorescent spirilla similar to *Methanospirillum hungatei*. In the marine cultures MaPim and CuPim, slightly curved rods predominated which partly formed spores, and resembled *Syntrophospora bryantii* ('*Clostridium bryantii*') [13] (Fig. 1c). The marine enrichment cultures with adipate (MaAd, CuAd) did not grow any further after the second transfer, and were discarded. Isolation of adipate- or pimelate-degrading bacteria in pure culture or in defined binary cultures with hydrogenotrophic bacteria (*Desulfovibrio vulgaris* or *Methanospirillum hungatei*) by agar dilution series was attempted repeatedly, but failed.

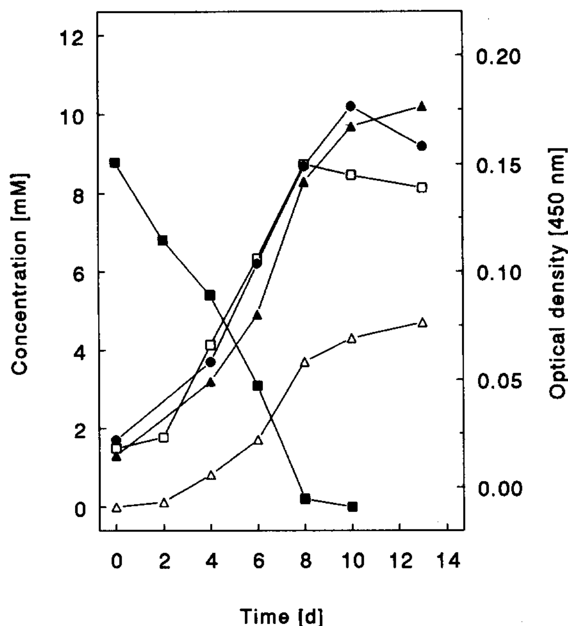

Fig. 2. Anaerobic degradation of adipate (■) by the enrichment culture WoAd. Other symbols refer to: optical density at 450 nm (ΔOD_{450}; □), propionate (●), acetate (▲), and methane (△).

Degradation of adipate and pimelate by methanogenic enrichment cultures

Adipate was completely degraded within 8–10 days by the highly enriched cultures KoAd and WoAd. Nearly equimolar amounts of propionate and acetate were formed, concomitant with methane production and growth (Fig. 2, Table 1). Compared to propionate formation, propionate degradation to acetate and methane was very slow. No valerate was formed in these cultures.

Table 1

Degradation stoichiometries of enrichment cultures

Enrichment culture	Substrate	Substrate consumed (μmol)	Products formed (μmol)				Electron recovery (%)
			Acetate	Propionate	Butyrate	Methane	
WoAd	Adipate	440	420	425	0	215	96
KoAd	Adipate	395	385	380	0	210	98
MaPim	Pimelate	490	1445	0	0	575	103
WoPim	Pimelate	500	1020	0	190	320	91
MaPim	Suberate	355	735	315	0	475	104
MaPim	Azelate	420	1300	0	0	1200	108
MaPim	Sebacate	545	895	450	0	1375	90

An adipate-pregrown culture WoAd grew also with succinate or pimelate without any lag phase. From succinate, propionate was formed as an intermediate in addition to acetate and methane, whereas butyrate was formed from pimelate. Both fatty acids were further transformed to acetate, and methane was formed.

Pimelate was completely degraded within 12–15 days by both enrichment cultures, WoPim and MaPim. Culture WoPim grew with pimelate with concomitant formation of acetate, butyrate, and methane (Fig. 3a, Table 1). Butyrate accumulated to a concentration of 4 mM and was quickly transformed further to acetate. In the marine culture MaPim, pimelate was degraded exclusively to acetate and methane, and no butyrate accumulated intermediately (Fig. 3b, Table 1). Caproate was never formed, neither by the culture WoPim nor by the culture MaPim. A pimelate-pregrown culture MaPim grew also with glutarate or adipate forming also acetate and methane without any lag phase. With adipate as substrate, propionate was an additional product.

In fermenting cultures, adipate and pimelate degradation depended strictly on the activity of hydrogenotrophic methanogens. Addition of 30 mM bromoethanesulfonate (BES), a specific inhibitor of methanogenesis [14], strongly inhibited adipate or pimelate degradation in dense cell suspensions of KoAd, WoPim, or MaPim. Adipate and pimelate degradation rates were decreased to 6–18% of the original rates in the absence of BES. Concomitant with inhibition of pimelate degradation, acetate and methane formation also stopped almost completely in the presence of BES, as shown in Fig. 4.

Degradation of suberate, azelate and sebacate by a methanogenic enrichment culture

The highly enriched culture MaPim which was obtained with pimelate as substrate grew also with suberate (C_8), azelate (C_9), and sebacate (C_{10}). Decomposition of these substrates was coupled to formation of acetate and methane. With azelate, no further product was detected. From suberate (7 mM) and sebacate (12 mM), propionate was formed at about a 1:1 ratio to the substrate provided (Table 1).

Discussion

In this paper, we document for the first time methanogenic degradation of long-chain dicar-

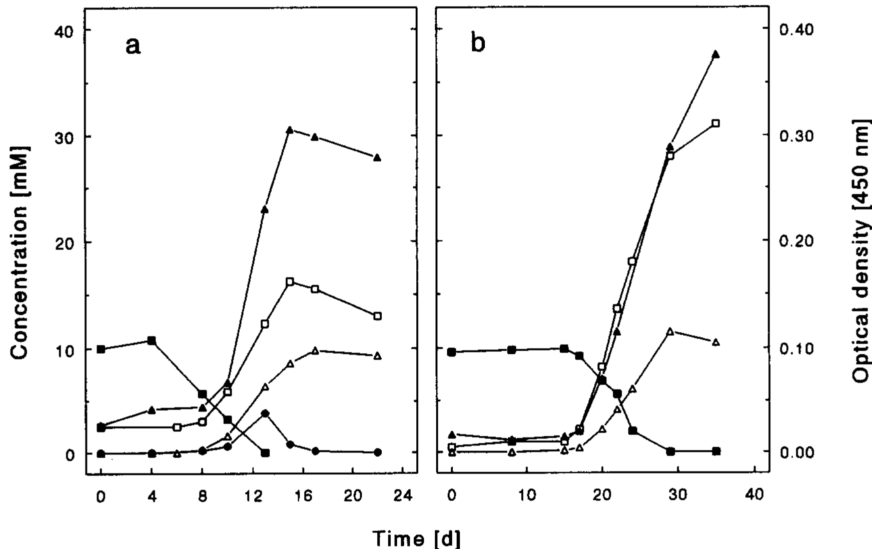

Fig. 3. Anaerobic degradation of pimelate (■) by the enrichment cultures WoPim (a) and MaPim (b). Other symbols refer to: optical density at 450 nm (ΔOD_{450}, □), butyrate (●), acetate (▲), and methane (△).

Fig. 4. Inhibition of pimelate (□, ■) degradation, acetate (○, ●) and methane (△, ▲) formation in cell suspensions of the enrichment culture MaPim (75 μg protein ml^{-1}) by 30 mM bromoethanesulfonate (BES). Open symbols refer to the presence of BES, closed symbols to BES-free controls.

boxylic acids of chain lengths C_6–C_{10} by enrichment cultures. Concomitant with the consumption of adipate, pimelate, suberate, azelate or sebacate, acetate and methane were formed, but no valerate or caproate. Cell suspension experiments with bromoethanesulfonate (BES) clearly showed that degradation of adipate and pimelate depended strictly on the activity of hydrogenotrophic methanogenic bacteria. These results indicate that the primary attack on these dicarboxylates is β-oxidation rather than decarboxylation. Thus, in contrast to short-chain dicarboxylic acids, dicarboxylates of more than 5 carbon atoms are first oxidized through syntrophic β-oxidation similar to fatty acids [15] until a chain length is obtained that allows biochemical decarboxylation (Fig. 5).

During β-oxidation of even-numbered dicarboxylates, propionate was formed as byproduct, together with acetate. Obviously, a succinyl residue was decarboxylated to a propionyl residue in these cultures indicating that decarboxylation occurred with the C_4-dicarboxylate, probably through the methylmalonyl-CoA pathway [16]. Obviously, further propionate oxidation is carried out by a specialist (e.g. *Syntrophobacter wolinii* [17]) which had been outdiluted during our enrichment procedure.

In enrichment cultures with odd-numbered long-chain dicarboxylates, acetate was formed as the only acid product; in freshwater enrichment cultures with pimelate, butyrate accumulated intermediately in addition to acetate. These substrates were probably β-oxidized to a glutaryl residue which can be decarboxylated after dehydrogenation to glutaconyl-CoA [7,18]. The resulting crotonyl-CoA is either further oxidized to two acetyl residues or reduced to butyrate.

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Fig. 5. Simplified scheme of methanogenic degradation of long-chain dicarboxylic acids.

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