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Anaerobic oxidation of fatty acids by *Clostridium bryantii* sp. nov., a sporeforming, obligately syntrophic bacterium

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Abstract. From marine and freshwater mud samples strictly anaerobic, Gram-positive, sporeforming bacteria were isolated which oxidized fatty acids in obligately syntrophic association with H_2-utilizing bacteria. Even-numbered fatty acids with up to 10 carbon atoms were degraded to acetate and H_2, odd-numbered fatty acids with up to 11 carbon atoms including 2-methylbutyrate were degraded to acetate, propionate and H_2. Neither fumarate, sulfate, thiosulfate, sulfur, nor nitrate were reduced. A marine isolate, strain CuCa1, is described as type strain of a new species, *Clostridium bryantii* sp. nov.

Key words: *Clostridium bryantii* sp. nov. species description – Fatty acid degradation – Anaerobic fermentation – Obligate syntrophy – Methanogenesis

Long-chain fatty acids are constituents of fats and lipids whereas short-chain fatty acids are formed as products of anaerobic fermentations (Thauer et al. 1977). In the presence of oxygen, fatty acids are completely degraded by β-oxidation via acetyl-CoA units. Under anaerobic conditions, fatty acid oxidation probably occurs in a similar manner. The reducing equivalents obtained during β-oxidation are either used for reduction of inorganic electron acceptors such as sulfate (Pfennig et al. 1981) or have to be released as molecular hydrogen. Since the release of hydrogen in this process is thermodynamically unfavorable, fatty acid-oxidizing acetogenic bacteria can only be cultivated in coculture with hydrogen-utilizing partners which keep the hydrogen partial pressure low (Thauer et al. 1977).

Syntrophic associations of fatty acid-degrading anaerobes have recently been described. *Syntrophomonas wolfei* is a motile Gram-negative rod which degrades fatty acids with 4 to 8 carbon atoms to acetate, propionate and hydrogen in coculture with hydrogen-scavenging sulfate reducers or methanogens (McInerney et al. 1979). *Syntrophobacter wolinii* only converts propionate to acetate, hydrogen and carbon dioxide or formate (Boone and Bryant 1980).

We now report on the isolation and properties of an anaerobic sporeforming bacterium degrading fatty acids with 4 to 11 carbon atoms in coculture with hydrogen-utilizing anaerobes.

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Materials and Methods

Sources of isolates

The following strains were isolated in coculture with either *Methanospirillum hungatei* or *Desulfovibrio* sp. from anaerobic enrichment cultures inoculated with mud samples:

Strain CuCa1 from marine anoxic mud samples taken near Cuxhaven, FRG, WoCa1 from mud of a creek near Konstanz, strain KoCa2 from anaerobic digestor sludge of the municipal sewage treatment plant, Konstanz, FRG. *Desulfovibrio vulgaris* strain Marburg was kindly provided by Prof. Dr. R. K. Thauer, Marburg.

Desulfovibrio sp. strain E70 was isolated by Dr. F. Widdel, Konstanz, from anoxic marine sediments with H_2/CO_2 and acetate. It utilizes lactate, pyruvate, and formate as substrates for growth.

Methanospirillum hungatei strain M1h was isolated from digested sludge of the municipal sewage plant, Göttingen. It utilizes H_2/CO_2 or formate as growth substrates.

All procedures for cultivation as well as for analysis of metabolic products were carried out as previously described (Widdel and Pfennig 1981; Schink and Pfennig 1982; Stieb and Schink 1984). The mineral medium for enrichments and further cultivation was carbonate-buffered and sulfide-reduced and contained the trace element solution SL 9 (Tschech and Pfennig 1984). The pH was adjusted to 7.2–7.4. Freshwater medium contained 0.1% NaCl and 0.04% $MgCl_2 \cdot 6\,H_2O$ whereas salt water medium contained 2% NaCl and 0.3% $MgCl_2 \cdot 6\,H_2O$.

Isolation and characterization

Defined mixed cultures were obtained by repeated application of the agar shake culture method described by Pfennig (1978). 0.5 ml of a grown *Desulfovibrio* sp. culture was added to each tube before gassing with N_2/CO_2 (80%/20%) mixture. Purity was checked microscopically after growth in mineral medium with 10 mM caproate and 20 mM sulfate or in complex medium (AC-Medium, Difco Laboratories, Detroit, MI, USA) with or without 10 mM caproate and 20 mM sulfate. Growth experiments were carried out at 28° C. Because cells preferentially grew in flocs, growth could not be followed by optical density measurements. Therefore, metabolic activity was followed by gas chromatographic analysis of substrate degradation and product formation.

All chemicals were of reagent grade and were obtained from E. Merck AG, Darmstadt, FRG; Serva, Heidelberg, FRG; Fluka AG, Neu-Ulm, FRG.

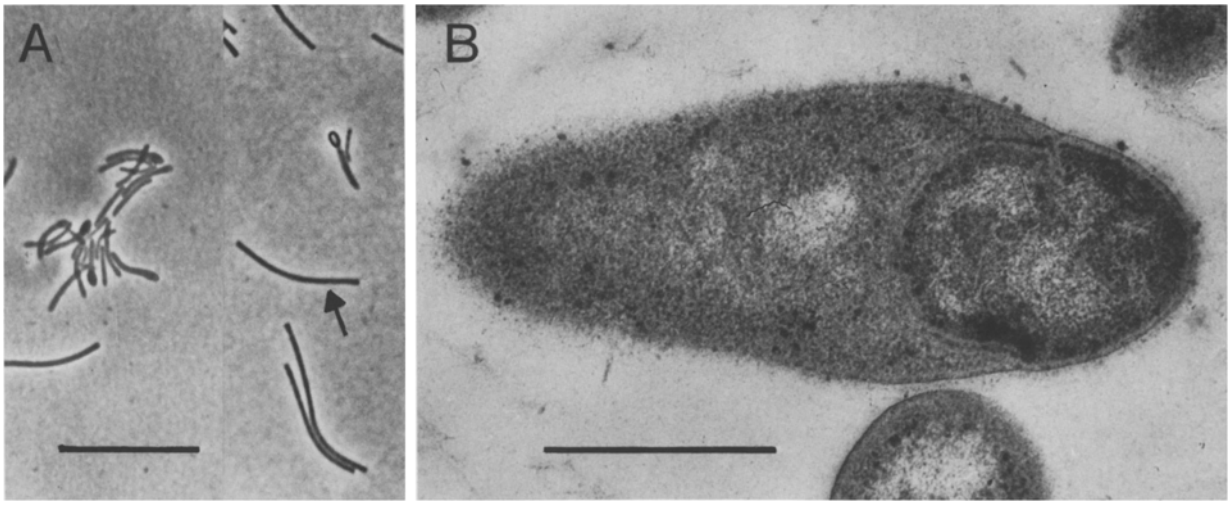

Fig. 1. A Phase contrast micrograph of sporulated cells of *Clostridium bryantii* strain CuCa1 cocultured with *Methanospirillum hungatei*. Arrow points at *M. hungatei* cell. Bar represents 10 μm. **B** Ultrathin section of a sporulating cell of *Clostridium bryantii* strain CuCa1. Bar represents 0.5 μm

Results

Enrichment, isolation and enumeration

Enrichment cultures with 10 mM caproate in either freshwater or salt water medium were inoculated with 5 ml of anoxic mud from various habitats. Gas production started after 2–3 weeks indicating substrate degradation. After several transfers, the subcultures contained mainly nonmotile sporeforming rods and rod-shaped fluorescent methanogens. Enrichment cultures with caproate as sole substrate formed acetate and methane as fermentation products.

Isolation was tried in agar shake cultures in mineral medium containing 10 mM caproate, 20 mM sulfate, 5 mM acetate and *Desulfovibrio* sp. Yellow, disk-shaped colonies appeared after 3 weeks of incubation. After two subsequent dilution series in agar shake cultures, defined mixed cultures could be isolated (strains CuCa1, WoCa1 and KoCa2).

Enumeration of caproate-oxidizing bacteria was carried out by the three-tube most probable number technique (American Public Health Association 1969) in 2 muds of different origin. The medium contained *Methanospirillum hungatei* as hydrogen scavenger. Tubes were checked for growth by assessment of turbidity and methane formation. 100,000 cells per ml were found in the mud sample of the creek near Konstanz, and 460 cells per ml in the Cuxhaven sediment sample. This is in accordance with the assumption that in a marine sediment sulfate-reducing bacteria are the primary fatty acid oxidizers. The prevalent caproate-oxidizing anaerobes found in the highest positive dilution tubes were morphologically similar to strains WoCa1 and CuCa1.

All strains were rod-shaped, sporeforming and nonmotile. Substrate utilization was tested with strains CuCa1 and WoCa1. Both strains degraded the same substrates. Because strains WoCa1 and KoCa1 did not grow well on mineral medium, strain CuCa1 was chosen for further characterization.

Characterization of strain CuCa1

Morphology and cytological properties. Cells of strain CuCa1 were slender, slightly curved, non-motile rods with rounded ends, $0.4 \times 3-6$ μm in size, occurring singly or in clumps (Fig. 1 A). The Gram reaction was negative to weakly positive. No indication of an outer cell membrane as typical of Gram-negative bacteria could be found in ultrathin sections of sporulating cells (Fig. 1 B).

Oval spores, 0.75×1.5 μm in size, were formed at the cell ends after at least 4 weeks of incubation. After longer incubation periods spores could also be detected free in the medium. Spores were heat-resistant and survived pasteurization (15 min at 80° C).

Nutritional properties and growth characteristics of strain CuCa1. Strain CuCa1 grew well in coculture with either *Methanospirillum hungatei* or *Desulfovibrio* sp. in mineral medium with 10 mM caproate and at least 1% NaCl and 0.15% $MgCl_2 \cdot 6\,H_2O$. Vitamins were present in the medium, however, were not necessary for growth in salt water medium for at least 5 subsequent transfers. No growth was found under aerobic conditions. Phosphate completely inhibited growth at concentrations higher than 10 mM.

The only substrates utilized were fatty acids with 4–11 carbon atoms including 2-methylbutyrate. Other substrates such as propionate, fatty acids with 12–18 carbon atoms, isobutyrate, 3-methylbutyrate, 3-hydroxybutyrate, crotonate, acetoacetate ethyl ester, pyruvate, lactate, succinate, fumarate, glycerol, glucose, fructose, ribose or yeast extract were not metabolized. Utilization of electron acceptors was tested with cultures inoculated with pasteurized sporulated cells. Neither fumarate nor sulfate, thiosulfate, sulfur or nitrate was reduced. Growth was observed at 28° C and 34° C, but not at 20° C and 40° C. The pH limits were pH 6.5 and 7.5. Substrate degradation and product formation during growth on caproate under optimal conditions is presented in Fig. 2. Independently of the hydrogen-utilizing partner, butyrate was excreted during caproate degradation, and was utilized later on. The doubling time as estimated from acetate formation rates was about 72–96 h in both cases, however, cocultures with *Desulfovibrio* sp. had shorter lag periods after transfer than those with *M. hungatei*.

The relationship between substrate utilization and product formation is given in Table 1. Even-numbered fatty acids were degraded to acetate and sulfide or methane, whereas

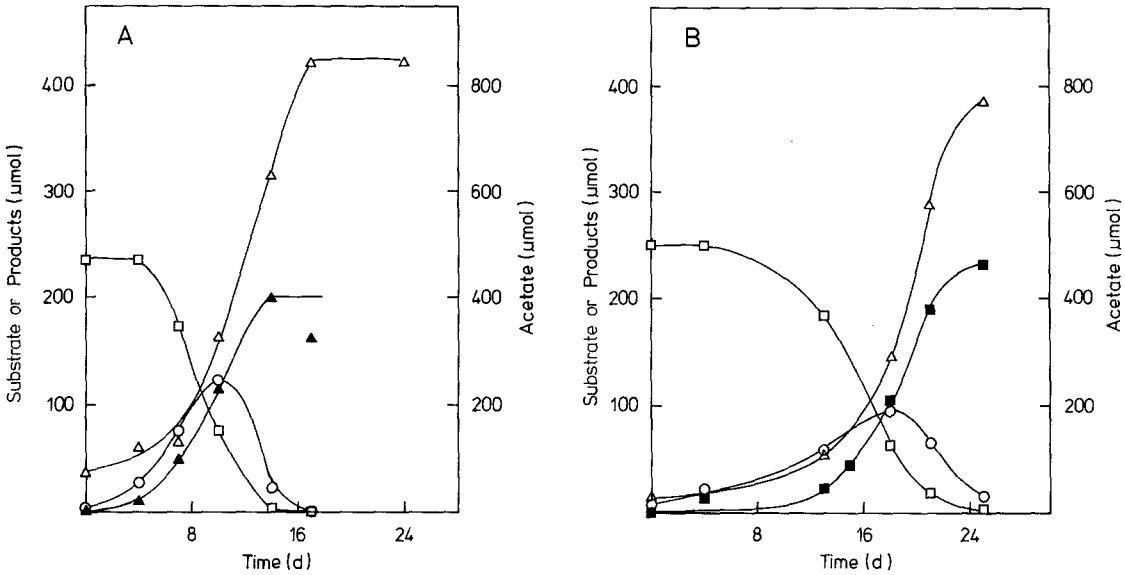

Fig. 2. Time course of caproate degradation and product formation by *Clostridium bryantii* strain CuCa1, **A** in coculture with *Desulfovibrio* sp., **B** in coculture with *Methanospirillum hungatei*. □, caproate; △, acetate; ○, butyrate; ▲, sulfide; ■, methane

Table 1. Stoichiometry of fermentation by strain CuCa1 in coculture with either *Desulfovibrio* sp. strain E70 or *Methanospirillum hungatei*. Experiments were carried out in screw cap tubes with butyl rubber septa

Substrate	No. of C-atoms	Amount of substrate degraded [µmol]	Products [µmol]			
			Acetate	Proprionate	HS^- [a]	CH_4 [b]
Butyrate	4	200	398		75	
Butyrate	4	100	184			53
Valerate	5	200	216	183	76	
Valerate	5	100	104	104		56
2-Methylbutyrate	5	200	204	194	131	
Caproate	6	200	613		163	
Caproate	6	200	590			182
Heptanoate	7	100	200	77	73	
Caprylate	8	20	78		34	
Caprylate	8	10	36			14
Pelargoate	9	20	68	19	30	
Caprinate	10	20	126			
Undecanoate	11	20	95	19	38	

[a] In coculture with *Desulfivibrio* sp.
[b] In coculture with *Methanospirillum hungatei*

odd-numbered fatty acids including 2-methylbutyrate were degraded to acetate, propionate and sulfide or methane. The stoichiometry of product formation is in accordance with the following fermentation equations for degradation of caproate in coculture with either *Desulfovibrio* sp. or *Methanospirillum hungatei:*

$$C_6H_{11}O_2^- + SO_4^{2-} \rightarrow 3\,C_2H_3O_2^- + HS^- + H^+$$
$$\Delta G_o' = -58.3 \text{ kJ/mol}$$

$$C_6H_{11}O_2^- + H_2O + HCO_3^- \rightarrow 3\,C_2H_3O_2^- + CH_4 + H^+$$
$$\Delta G_o' = -40.83 \text{ kJ/mol}$$

Gibbs free energies were calculated after published tables (Thauer et al. 1977).

Discussion

The bacteria described in the present study represent another species of obligately syntrophic fatty acid-oxidizing anaerobes similar to *Syntrophomonas wolfei* (McInerney et al. 1979, 1981). Similar to this species, our isolates are specialized for syntrophic oxidation of fatty acids and do not use any other kind of substrates. However, the range of substrates used by our isolates is broader than that reported for *S. wolfei* which only uses fatty acids up to 8 carbon atoms. Moreover, our isolates are able to degrade also 2-methylbutyrate, a product of anaerobic degradation of leucine (Barker 1981). The new strains appear to be better adapted to long-chain fatty acids than *S. wolfei*: Enrich-

ments and enumerations with caproate as substrate always yielded bacteria similar to those described in this paper whereas enrichments with butyrate as substrate always led to bacteria similar to *S. wolfei*. However, the range of utilizable fatty acids is limited also for our strains, and in spite of numerous efforts we so far did not succeed in isolation of syntrophic bacteria able to degrade long-chain fatty acids like palmitate or stearate. It appears that anaerobic degradation of fatty acids is subdivided into two different ranges, the short-chain and the long-chain range, and different types of bacteria are specialized for either one.

During degradation of caproate, our isolates excreted butyrate as an intermediate product which later on was oxidized to acetate. If medium-chain fatty acids are excreted during long-chain fatty acid degradation in a similar manner, they could well serve as substrates for our isolates.

Our isolates are the first obligately syntrophic anaerobes which are Gram-positive. Lack of an outer cell membrane poses the question on the localization of hydrogenases in these bacteria. So far, periplasmic hydrogenases were supposed to be useful, if not necessary for an efficient interspecies hydrogen transfer (Bell et al. 1974), and may even be one of the reasons why sulfate reducers can outcompete methanogens for hydrogen (Kristjansson et al. 1982). Our isolates provide evidence that also a different hydrogenase localization can be sufficiently competitive.

Unfortunately, our isolates were not able to grow on partly unsaturated fatty acids. Disproportionation of e.g. crotonate to acetate and butyrate would allow growth of these bacteria in pure culture independent of syntrophic partners. Enrichments with crotonate and 3-hydroxybutyrate recently led to the isolation of a new metabolically very versatile anaerobe which, however, did not participate in interspecies hydrogen transfer (Stieb and Schink 1984).

The ability of our isolates to form spores make these organisms very handy for transfer between different hydrogen-scavenging partners. Moreover, this may allow to cultivate this organism free of contaminating partners in membrane-separated mixed culture systems. Experiments in this field are in progress, however, were not successful so far.

Taxonomy

As obligately anaerobic, sporeforming, non-sulfate-reducing bacterium, our isolate has to be classified with the genus *Clostridium* sp. (Buchanan and Gibbons 1974). The range of substrates utilized and its obligately syntrophic dependence on H_2-scavenging partners distinguishes strain CuCa1 from all other *Clostridium* sp. Therefore, a new species, *Clostridium bryantii* is proposed.

C. bryantii sp. nov. bry. an′ ti. i. M. L. gen. n. named for Marvin P. Bryant, who pioneered studies on syntrophic methanogenic associations.

Rod-shaped cells, $0.4 \times 4.5-6$ µm in size with rounded ends. Non-motile, slightly curved. Gram reaction negative to weakly positive; no outer cell membrane. Spores terminal, oval, 0.75×1.5 µm in size.

Strictly anaerobic chemoorganotroph. Fatty acids with 4–11 carbon atoms and 2-methylbutyrate utilized for growth and fermented to acetate and H_2 or to acetate, propionate and H_2 in syntrophic association with hydrogen-scavenging anaerobes. No other organic acids, no sugars or alcohols metabolized. Sulfate, sulfur, thiosulfate, nitrate, or fumarate not reduced.

Selective enrichment in mineral medium with caproate as substrate. pH range: 6.5–7.5. Growth at 28° C and 34° C, not at 20° C and 40° C. Type strain: CuCa1, DSM 3014A, B (A: coculture with *Desulfovibrio* sp., B: coculture with *Methanospirillum hungatei*), deposited in Deutsche Sammlung von Mikroorganismen, Göttingen, FRG.

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