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Anaerobic degradation of isovalerate by a defined methanogenic coculture

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Abstract. Isovalerate-oxidizing strictly anaerobic bacteria were isolated from marine sediment and sewage sludge in coculture with *Desulfovibrio* sp. Cells stained Gram positive and behaved Gram positive also in Gram classification with KOH. Isovalerate degradation depended on interspecies hydrogen transfer to syntrophic hydrogen-oxidizing sulfate reducers or methanogens. Isovalerate was the only substrate utilized and was fermented to 3 mol acetate and 1 mol hydrogen per mol substrate. The degradation pathway was studied by enzyme assays in crude cell extracts, and included acetyl-CoA dependent activation of isovalerate, oxidation to methylcrotonyl-CoA and carboxylation to methylglutaconyl-CoA which is hydrated and cleaved to acetoacetate and acetyl-CoA. Studies with inhibitors and ionophores suggest that energy conservation with this organism depends on either acetate efflux-driven proton symport or on an ion-gradient driven carboxylation mechanism.

Key words: Syntrophic degradation – Methanogenesis – Fatty acid oxidation – Isovalerate – Energy metabolism

Degradation of the amino acids leucine, isoleucine and valine by deamination and oxidative decarboxylation leads to the formation of branched-chain fatty acid residues (Massey et al. 1976). These fatty acids are excreted by anaerobic amino acid-degrading bacteria (Barker 1981; Allison 1978; Harwood and Canale-Parola 1981; McInerney 1986) and their further fate in anaerobic environments is largely unknown. In some cases, e.g. in the rumen ecosystem, branched-chain fatty acids can be used again for amino acid synthesis by rumen microorganisms (Allison and Bryant 1963; Allison 1969). Some sulfate-reducing bacteria were isolated recently which are able to oxidize branched-chain fatty acids either incompletely to acetate residues or completely to carbon dioxide with concomitant reduction of sulfate to sulfide (Widdel 1980; Widdel and Pfennig 1984). In the absence of sulfate, neovalerate (2-methylbutyrate) can be oxidized to acetate and propionate by the fatty acid-oxidizing *Clostridium bryantii* in syntrophic cooperation with hydrogen-scavenging methanogenic bacteria (Stieb and Schink 1985).

The present study describes the enrichment, isolation, and characterization of anaerobic bacteria which degrade isovalerate (3-methylbutyrate) to acetate in syntrophic association with hydrogen-oxidizing anaerobes.

Materials and methods

Sources of organisms

From marine and freshwater mud samples isovalerate degrading obligately syntrophic bacteria were isolated with *Desulfovibrio* sp. as hydrogen-scavenging partner. Strain GraIval was isolated from marine anoxic sediment of Canal Grande, Venice, Italy. Strain GöIval was isolated from anoxic digestor sludge of the municipal sewage treatment plant at Göttingen, FRG. *Desulfovibrio vulgaris* strain Marburg DSM 2119 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 formate, lactate, pyruvate, fumarate, and succinate as growth substrates. *Methanospirillum hungatei* strain Mlh was isolated from digested sludge of the municipal sewage plant, Göttingen. It utilizes H_2/CO_2 or formate as only growth substrates. *Methanosarcina barkeri* strain Göttingen, was isolated by Dr. F. Widdel, Konstanz.

Cultivation and isolation

All procedures for cultivation as well as for analysis of metabolic products were essentially as previously described (Widdel and Pfennig 1981; Schink and Pfennig 1982; Stieb and Schink 1984). The mineral medium for enrichment and further cultivation was carbonate-buffered and sulfide-reduced and contained the trace element solution SL 10 (Widdel and Pfennig 1984). The pH was adjusted to 7.2–7.4. Freshwater medium contained 1 g NaCl and 0.4 g $MgCl_2 \times 6\,H_2O$, brackish water medium 10 g NaCl and 1.5 g $MgCl_2 \times 6\,H_2O$, salt water medium contained 20 g NaCl and 3 g $MgCl_2 \times 6\,H_2O$ per liter medium.

Defined mixed cultures were obtained by repeated application of the agar shake culture method described by Pfennig (1978). 0.5 ml of a *Desulfovibrio* sp. culture (strain Marburg with freshwater, strain E70 with salt water cultures) was added to each tube as hydrogen scavenger after dilution and before gassing the headspaces with N_2/CO_2 (80%/20%). Purity was checked microscopically.

The Gram type was determined according to Gregersen (1978) and to Magee et al. (1975). *Escherichia coli* and *Acetobacterium woodii* were used as controls. Flagella were stained according to Mayfield and Inniss (1977).

Cells were grown in mineral medium containing 5 mM isovalerate, 5 mM sulfate, and *Desulfovibrio* sp. Methanogenic cocultures were obtained by transfer into serum bottles containing mineral medium with isovalerate and

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Methanospirillum hungatei, but without sulfate. All growth experiments were carried out at 28° C.

Enzyme assays

All assays of enzyme activities were carried out with a Zeiss PM4 spectrophotometer at 25° C under strictly anaerobic conditions in rubber-sealed 1 cm cuvettes. Crude cell extracts were prepared in 20 mM Tris-HCl buffer, pH 7.5, by French press treatment at 1750 bar and subsequent centrifugation at 20,000 × *g* for 15 min in a Sorvall centrifuge.

Isovaleryl-CoA dehydrogenase activity was determined following dichlorophenolindophenol reduction (Green et al. 1954). Isovaleryl-CoA synthetase activity was assayed in a coupled test with isovaleryl-CoA dehydrogenase. Instead of isovaleryl-CoA, 20 μl of 0.1 M ATP were added. The reaction was started by addition of 2 μl 40 mM CoA. 3-Hydroxy-3-methylglutaryl-CoA lyase activity was determined as NADH-dependent formation of 3-hydroxybutyrate. The assay was carried out as follows: 20 μl NADH (10 mg/ml), 10 μl 1 M $MgCl_2 \times 6\,H_2O$, 10 μl 0.1 M dithiothreitol and 10 μl 3-hydroxybutyrate dehydrogenase (5 mg/ml) were added to 0.9 ml 0.1 M Tris-HCl buffer, pH 8.1. Cell extract was added after the cuvette was sealed and gassed with N_2. After incubation at 28° C for 15 min the reaction was started by addition of 5 μl 20 mM 3-hydroxy-3-methylglutaryl-CoA. *β*-ketothiolase activity was determined following absorption increase at 233 nm (Lynen and Ochoa 1953). Phosphate acetyl transferase activity was determined following acetyl-CoA formation (Bergmeyer 1963). Acetate kinase activity was determined as NADH-dependent pyruvate reduction in a coupled test with pyruvate kinase and lactate dehydrogenase (Rose et al. 1954). Hydrogenase activity was determined with benzyl viologen as electron acceptor (Schink 1985). ATPase activity was determined as ADP-dependent conversion of phosphoenol pyruvate to pyruvate and lactate (Hilpert et al. 1984).

Acetyl-CoA: isovalerate CoA transferase activity was determined as isovalerate-dependent isovaleryl-CoA oxidation. Instead of isovaleryl-CoA, 20 μl 0.2 M isovalerate was added and the reaction was started by addition of 2 μl 20 mM acetyl-CoA. Acetoacetyl-CoA: acetate CoA transferase activity was determined by two different procedures. Either the decrease of acetyl-CoA was followed in arsenate buffer with 10 μl 1 M acetate and 2 μl 20 mM acetoacetyl-CoA (modified after Hilpert et al. 1984) or NADH decrease was followed in a reaction mixture containing 10 μl 1 M acetate, 20 μl NADH (10 mg/ml) and 5 μl 3-hydroxybutyrate dehydrogenase (5 mg/ml) in 0.9 ml 10 mM arsenate buffer, pH 7.0 with 52 mM KCl. Cell extract was added when the cuvette was sealed and gassed with N_2. The reaction was started by addition of 2 μl 20 mM acetoacetyl-CoA.

All chemicals were of reagent grade and were obtained from E. Merck AG, Darmstadt; Fluka AG, Neu-Ulm; Serva, Heidelberg. Biochemicals and enzymes were purchased from Sigma Chem. Co., München, and Boehringer, Mannheim, FRG.

Results

Enrichment, isolation and enumeration

50 ml enrichment cultures with 5 mM isovalerate in either freshwater or saltwater medium were inoculated each with 5 ml of anoxic mud from various habitats. Substrate-dependent gas production started after 3–4 weeks. Transfers into fresh medium were carried out when methane evolution ceased. The bacteria grew very slowly and were transferred at monthly intervals. After several transfers, the subcultures contained mainly thick rods which appeared highly refractile in phase contrast microscopy, and short, rod-shaped fluorescent methanogens. Acetate and methane were the only fermentation products detected.

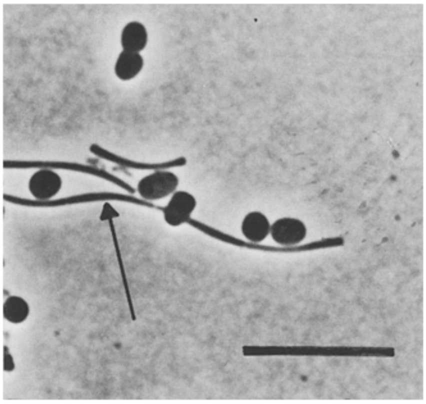

Fig. 1. Phase contrast photomicrographs of strain GraIval in coculture with *Methanospirillum hungatei* (*arrow*). Bar equals 10 μm

Isovalerate-fermenting bacteria were isolated in defined medium in coculture with *Desulfovibrio* sp. or by applying the agar shake culture method 5 times in mineral medium containing 10 mM isovalerate and 5 mM sulfate. Most of the colonies which appeared after 2 weeks contained sulfate-reducing cocci or rods similar to those observed in the enrichment cultures. These bacteria were often isolated instead of the syntrophic isovalerate oxidizing ones. Yellow, disc-shaped colonies which appeared after almost 3 weeks of incubation contained mixed cultures of rod-shaped bacteria and *Desulfovibrio* sp. These colonies were transferred into liquid media.

Enumeration of isovalerate-oxidizing bacteria was carried out by the three tube most probable number technique (American Public Health Association 1969) with two mud samples. *Methanospirillum hungatei* was added as hydrogen scavenger. After incubation for 2 months, tubes were checked for bacterial activity by assessment of turbidity and methane formation. 4.6×10^4 cells per ml were found in a mud sample of Rio Marin, Venice, and lower numbers in digested sludge of the municipal sewage plant, Konstanz (240 cells per ml). In both cases, the same type of refractile rods predominated which was isolated from the enrichment cultures.

Two strains of isovalerate-degrading bacteria which were morphologically similar were isolated in coculture with *Desulfovibrio* sp. The marine strain GraIval grew better in defined isovalerate medium than the freshwater strain GöIval, and was therefore chosen for further characterization. Better growth with strain GöIval was achieved when the bacteria were grown in brackishwater or saltwater.

Characterization of strain GraIval

Morphology and cytological properties

Cells of strain GraIval were thick rods, 1.0–1.25 μm × 1.75–2.5 μm in size with rounded ends, occurring singly or in clumps (Fig. 1). Dark cell inclusions observed

Desulfococcus multivorans (Widdel 1980; Widdel and Pfennig 1984); defined methanogenic cocultures growing with isobutyrate have not yet been described. *Desulfococcus multivorans* also oxidizes isovaleric acid (3-methylbutyrate; Widdel and Pfennig 1984) completely to carbon dioxide with concomitant reduction of sulfate to sulfide. The pathway of isovalerate degradation by this organism has not yet been studied.

In the present study, defined cultures of fermenting bacteria are described which oxidize isovalerate to acetate in syntrophic association with either sulfate-reducing or methanogenic bacteria. These bacteria were extremely difficult to enrich and to isolate. Contaminating sulfate-reducing isovalerate oxidizers similar to *D. multivorans* were carried over during transfers in the enrichment process; they probably could utilize oxidized sulfur compounds as electron acceptors which form from the sulfide added as reducing agent to the medium. Isolation of fermenting bacteria which cooperated with methanogenic bacteria required excessively long incubation times, and the isolated cocultures grew only very slowly ($t_d = 5-6$ days) and to only low cell densities. Since no other substrate but isovalerate was used, the study and cultivation of these bacteria posed extreme difficulties which can be explained to some extent on the basis of thermodynamic considerations.

The fermentation of isovalerate to acetate and hydrogen is an endergonic reaction under standard conditions (calculations after Thauer et al. 1977):

$$C_5H_9O_2^- + HCO_3^- + H_2O \rightarrow 3\,C_2H_3O_2^- + H_2 + H^+. \quad (3)$$

$$\Delta G_0{}' = +20.2 \text{ kJ per mol}$$

The oxidation of isovalerate to acetate becomes exergonic only if one of the reaction products, e.g. hydrogen, is used up by a partner organism. Thus, hydrogen oxidation by a methanogenic bacterium makes the overall reaction exergonic [$\Delta G_0{}' = -13.6$ kJ per mol isovalerate; Eq. (1)], and slightly more energy is available if sulfate is concomitantly reduced to sulfide [$\Delta G_0{}' = -17.8$ kJ per mol isovalerate; Eq. (2)]. The Gibbs free energy of ATP hydrolysis is in the range of 48 kJ per mol (Thauer et al. 1977); thus, at least 4 mol of isovalerate have to be oxidized to allow synthesis of 1 mol ATP, and this small amount of energy has to be shared by two organisms. The energetic situation of the isovalerate-degrading methanogenic coculture is distinctly worse than that of the butyrate and propionate-oxidizing systems (McInerney et al. 1979; Boone and Bryant 1980). Perhaps the most intriguing fact about these cultures is that growth is possible under these conditions at all, and it is understandable from this point of view that the growth rates and yields are extremely small. Theoretically, reaction (3) can be exergonic too if it is combined with the cleavage of acetate by an acetate-degrading methanogen:

$$C_5H_9O_2^- + 4\,H_2O \rightarrow 3\,CH_4 + 2\,HCO_3^- + H^+ + H_2. \quad (4)$$

$$\Delta G_0{}' = -71.6 \text{ kJ per mol}$$

This „interspecies acetate transfer" would represent a new type of syntrophic cooperation between anaerobic bacteria in which acetate has to be kept at minimum concentrations and hydrogen accumulates. Acetate removal is especially important in isovalerate degradation since three times as much acetate as hydrogen is formed (reaction 3). Our hydrogen-dependent methanogenic coculture was inhibited by the accumulation of acetate and did not degrade isovalerate beyond an initial concentration of 5 mM. If acetate was removed by *Methanosarcina barkeri*, also higher initial substrate concentrations were tolerated.

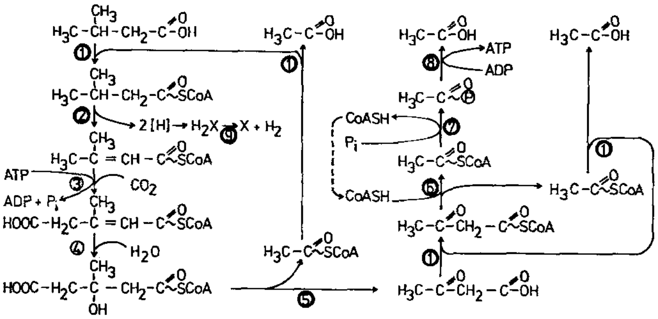

Fig. 3. Hypothetical pathway of isovalerate degradation by strain GraIval. Numbers refer to the following enzymes: *1* Acyl-CoA: acetate CoA transferase; *2* Acyl-CoA dehydrogenase; *3* methylcrotonyl-CoA carboxylase; *4* 3-methyl-glutaconyl-CoA hydratase; *5* 3-hydroxy-3-methylglutaryl-CoA lyase; *6* acetyl-CoA acyl transferase; *7* phosphate acetyl transferase; *8* acetate kinase; *9* hydrogenase

The pathway of isovalerate degradation by strain GraIval was studied by enzyme assays in crude cell extracts of the methanogenic coculture. Although some of these enzymes can also be involved in amino acid synthesis, in most cases their activities are high enough to account for isovalerate catabolism. On the basis of the enzyme activities detected, a degradation pathway is suggested (Fig. 3) which is similar to that established for aerobic isovalerate degradation (Lynen et al. 1961). Three mol of acetate and one mol of hydrogen gas are formed, while CO_2 is incorporated in the carboxylation of methylcrotonyl-CoA. Only the release of one acetate molecule can be coupled with ATP formation via acetate kinase; the two other acetyl-CoA residues formed are used for activation of isovalerate and 3-hydroxybutyrate in acyl transferase reactions. If the carboxylation of methylcrotonyl-CoA were coupled with the hydrolysis of one mol ATP per mol substrate, the overall ATP balance of the reaction sequence would be zero and no net energy could be conserved by substrate level phosphorylation. Since electron transfer reactions to allow electron transport phosphorylation are lacking, the way of energy conservation in this organism remains enigmatic. Physiological observations led to propose the following two hypotheses to explain the energy metabolism of the isovalerate-degrading bacteria: i) Excretion of the fermentation product acetate may be coupled to a cotransport of protons in a similar manner as observed with lactate excretion by *Streptococcus cremoris* (Michels et al. 1979; Konings and Veldkamp 1983). The proton gradient thus established could drive ATP synthesis by a proton-driven ATPase. Energy conservation based on such a mechanism should be abolished by proton ionophores. Our experiments showed that the proton ionophore CCCP inhibited growth completely at 1 µM concentration whereas the potassium ionophore valinomycin had no effect up to 10 µM concentration.

ii) Carboxylation of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA (reaction 3; Fig. 3) is catalyzed by a membrane-bound enzyme which obtains the necessary energy (around 17 kJ) not directly from ATP hydrolysis but from a sodium gradient established by a sodium ion-dependent ATPase. Carboxylation of carboxylic acid derivatives at the expense of sodium ion gradients has been demonstrated recently for the oxaloacetate decarboxylase of *Klebsiella aerogenes* (Dimroth and Hilpert 1984) and the methylmalonyl-CoA decarboxylase of *Propionigenium modestum* (Schink and Pfennig 1982; Hilpert et al. 1984). Carboxylation of 3-methylcrotonyl-CoA to 3-methylglutaconyl-CoA by such an ion transport system would require only a fraction of the energy released during the hydrolysis of 1 ATP, and would leave a small but sufficient fraction of this energy for biosynthetic purposes. The observation that both the freshwater and the saltwater isolates described in the present study are highly dependent on sodium ions for growth, and that the sodium ionophore monensin inhibited growth at low (10 μM) concentrations support this hypothesis. However, experiments with ionophores do not provide conclusive evidence when applied to growing cells. Unfortunately, the extreme difficulties of producing enough cell material for biochemical studies so far precluded to carry out definitive experiments with cell-free extracts.

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