

Anaerobe



METABOLISM/MICROBIAL CHEMISTRY

Utilization of Serine, Leucine, Isoleucine, and Valine by *Thermoanaerobacter brockii* in the Presence of Thiosulfate or *Methanobacterium* sp. as Electron Acceptors

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Thermoanaerobacter brockii fermented serine to acetate and ethanol. It oxidized leucine to isovalerate, isoleucine to 2-methylbutyrate, and valine to isobutyrate only in the presence of thiosulfate, or when co-cultured with *Methanobacterium* sp. This oxidative deamination was rendered thermodynamically possible by the ability of *T. brockii* to reduce thiosulfate to sulfide or the transfer of reducing equivalents to the hydrogenotrophic methanogen. The results suggest that *T. brockii* may be of ecological significance in thermal environments in the turnover of amino acids, especially with thiosulfate or H₂-utilizing methanogens are present.

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Introduction

Amino acid utilization amongst anaerobes has been widely studied in the genus *Clostridium* [1-4] but other

anaerobes of the genera *Peptococcus*, *Campylobacter*, *Megasphaera*, *Acidaminobacter*, *Eubacterium* or *Synergistes* are also known to utilize amino acids [5-10]. If chemical or biological electron acceptors are present, amino acids can be oxidized. The hydrogen produced from amino acid oxidation can be utilized by methanogens in various environments suggesting an important role of interspecies hydrogen transfer in the degradation of proteinaceous compounds [8,10-13].

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Members of the genera *Desulfovibrio* and *Desulfotomaculum* oxidize various single amino acids — including L-alanine, serine and glycine — if sulfate is present [14,15], whereas the hyperthermophilic sulfur reducing members of the order *Thermococcales*, domain *Archaea* can grow on mixtures of amino acids [16–18]. Some *Clostridium* species can also use amino acids using Stickland's reaction, one amino acid acting as electron donor and the other as electron acceptor [1].

Thermoanaerobacter brockii (formerly *Thermoanaerobium brockii* [19]) was first described as not using amino acids [19]. However, we recently reported that *Thermoanaerobacter* species including *T. brockii* oxidized these compounds in the presence of thiosulfate to produce acetate, isobutyrate, isovalerate, traces of propionate, and hydrogen sulfide [20]. We have now identified the amino acids involved and provide evidence that they can also be oxidized in the presence of a *Methanobacterium* strain which acts as a biological electron acceptor.

Materials and Methods

Thermoanaerobacter brockii HTD4^T (= DSMZ 1457^T) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. *Thermoanaerobacter* strain SEBR 5268 was isolated from water from an oil field and recognized as belonging to the species *T. brockii* [21,22]. The thermophilic hydrogenotrophic *Methanobacterium* sp. was isolated from a French oil field sample and is available from our culture collection. *Thermoanaerobacter* strains were grown in Hungate tubes in 5 mL of basal medium [21] modified to contain 2 g/L of yeast extract, and

dispensed anaerobically as described previously [23]. *Methanobacterium* sp. was cultured in the same basal medium but under a H₂ + CO₂ [80/20%, 2 bars (2 × 10⁵ Pa)] gas phase. Twenty mM amino acids (DL-tyrosine, L-lysine, L-alanine, DL-serine, L-isoleucine, DL-glycine, glutamate, L-glutamine, L-methionine, L-arginine, L-cysteine, DL-proline, DL-tryptophan, DL-threonine, DL-phenylalanine, DL-aspartate, DL-histidine, DL-valine, DL-leucine, and L-asparagine) and 20 mM thiosulfate were added to the prereduced basal medium from sterile anaerobic solutions. Experiments were duplicated. Volatile fatty acids and H₂ were analysed as described previously [21]. 2-methylbutyrate was analysed on a gas chromatograph equipped with a flame ionization detector using a silica capillary column at 80°C [(30 m × 0.25 mm, optima 1701–0.50 μm), Machery Nagel, Germany]. Growth was measured by inserting tubes directly into a model UV-160A spectrophotometer (Shimadzu Corp., Kyoto, Japan) and measuring the optical density at 580 nm. Sulfide was determined by the method of Cord–Ruwisch [24]. Amino acids were measured by high pressure liquid chromatography [25].

Results and Discussion

Thermoanaerobacter brockii oxidized leucine, isoleucine, and valine only if thiosulfate was present (Table 1). In contrast, serine was utilized in the absence of thiosulfate, which shows that it could be fermented (Table 2). None of the other amino acids tested was used either in the presence or in the absence of thiosulfate. Similar results were obtained with *Thermoanaerobacter* strain SEBR 5268 (data not shown). Addition of

Table 1. Utilization of leucine, isoleucine and valine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methanobacterium* sp.

Culture conditions	Products formed (mM) ^{a,b}							amino acid degraded (mM)
	Maximal O.D. (580nm)	Isobutyrate	Isovalerate	2-methyl butyrate	H ₂ S	H ₂	CH ₄	
Control	0.163 ± 0.050	0.1	0.1	0.1	0.5	1.8	0.0	—
Control + thiosulfate	0.180 ± 0.000	0.3	1.2	0.3	0.8	0.2	0.0	—
Control + <i>Methanobacterium</i>	0.185 ± 0.050	0.5	1.6	0.4	0.3	1.5	0.8	—
Leucine	0.100 ± 0.022	0.3	1.3	0.2	0.5	3.7	0.0	0.2
Leucine + thiosulfate	0.242 ± 0.030	0.3	23.5	0.4	15.5	1.1	0.0	22.5
Leucine + <i>Methanobacterium</i>	0.215 ± 0.013	1.2	22.7	0.3	0.3	0.0	8.0	17.0
Isoleucine	0.140 ± 0.030	0.4	0.2	1.9	0.6	5.0	0.0	0.8
Isoleucine + thiosulfate	0.229 ± 0.003	0.3	1.5	19.1	34.5	2.2	0.0	32.3
Isoleucine + <i>Methanobacterium</i>	0.267 ± 0.020	1.2	1.5	19.9	0.3	0.0	9.2	20.5
Valine	0.191 ± 0.008	0.4	0.9	0.1	0.5	3.0	0.0	0.0
Valine + thiosulfate	0.146 ± 0.001	9.0	1.4	0.5	1.5	0.5	0.0	15.0
Valine + <i>Methanobacterium</i>	0.214 ± 0.030	11.0	1.7	0.6	0.3	0.0	4.6	13.6

^aResults obtained after incubation at 62°C.

^bAcetate concentration did not change after adding amino acid in the absence or presence of an electron acceptor as compared to the respective control.

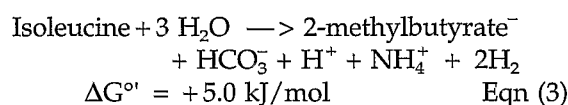
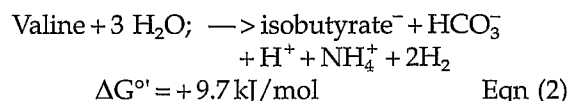
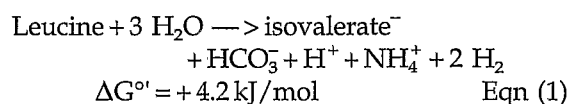
leucine or isoleucine in the culture medium slightly improved *T. brockii* growth [Table 1, Figure 1(a)] indicating that ATP was generated from their oxidation in the presence of thiosulfate. This was in accordance with the change in Gibbs free energy, under standard conditions for the degradation of these amino acids, resulting from reducing equivalents removal [26]. In contrast, the addition of valine decreased *T. brockii* growth [Table 1, Figure 1(b)]. Therefore the uptake of valine might be an ATP-requiring process similar to that reported for *Clostridium hydroxybenzoicum* grown on arginine [27].

The fermentation of serine to acetate and ethanol by *T. brockii* was similar to that reported for *Peptococcus prevotii* or *Eubacterium acidaminophilum* [10,28], and most likely occurred via serine dehydratase [28]. During the oxidation of serine in the presence of thiosulfate or *Methanobacterium* sp. as electron acceptors, sulfide or methane was produced respectively and no hydrogen was formed (Table 2). Furthermore, in both cases, ethanol concentration drastically decreased whereas the amount of acetate increased, indicating that the use of each electron acceptor caused a shift in the flow of electrons channelled away from ethanol to acetate. Such a shift in metabolism was previously reported for *Thermoanaerobacter* sp. when grown on glucose in the presence of thiosulfate [29].

Leucine was oxidized to isovalerate (3-methylbutyrate), isoleucine to 2-methylbutyrate, and valine to isobutyrate (Table 1). The metabolism of *T. brockii* therefore resembles that of several microorganisms from the domains *Archaea* and *Bacteria*, including *Clostridium* sp. [2], *Thermococcus stetteri* [30], *Thermoproteus uzoniensis* and *T. tenax* [31], *Staphylothermus marinus* [32], and *Pyrodicticum abyssi* [33]. All were reported to produce isobutyric and isovaleric acids from peptide and amino acid fermentation or oxidation, perhaps as a result of valine or leucine oxidation.

Thermoanaerobacter brockii degraded leucine, isoleucine, and valine and produced fatty acids that were

one carbon shorter than the corresponding amino acid. This indicates that these amino acids are most probably used via oxidative deamination with the oxo acid intermediate being decarboxylated [2,3,26] [equations (1–3)]



A good electron balance was obtained by taking into account the acid, and methane produced by the co-culture *T. brockii* – *Methanobacterium* sp. from leucine, isoleucine, and valine oxidation. In contrast, in the presence of thiosulfate, a low electron recovery was obtained when *T. brockii* was grown on leucine or valine. For example, when *T. brockii* was grown on valine in the presence of thiosulfate, the H_2S produced (1.5 mM) was less than expected from the amount of amino acid used (15 mM). Therefore, it can be suggested that an intermediary sulfur compound could accumulate during the reduction of thiosulfate by *Thermoanaerobacter brockii*. Experiments to identify the intermediary products are being conducted in our laboratory.

Our results also demonstrate that leucine, isoleucine, and valine were oxidized in the presence of the hydrogenotrophic methanogen, *Methanobacterium* sp. (Table 1). The decrease in H_2 concentration was higher when *T. brockii* was co-cultured with *Methanobacterium* sp. as compared with thiosulfate. This agrees with other results providing evidence that extensive amino acid or peptide degradation can occur during

Table 2. Utilization of serine by *Thermoanaerobacter brockii* in the presence of thiosulfate or *Methanobacterium* sp.

Culture conditions	Products formed (mM) ^{a,b}						amino acid degraded (mM)
	Maximal O.D. (580nm)	Acetate	Ethanol	H ₂ S	H ₂	CH ₄	
Control	0.163 ± 0.050	0.1	0.0	0.5	1.8	0.0	—
Control + thiosulfate	0.180 ± 0.000	0.4	0.0	0.8	0.2	0.0	—
Control + <i>Methanobacterium</i>	0.185 ± 0.050	0.3	0.0	0.3	1.5	0.8	—
Serine	0.172 ± 0.043	18.3	7.8	0.7	5.0	0.0	27.0
Serine + thiosulfate	0.220 ± 0.042	23.1	0.0	11.4	0.0	0.0	25.5
Serine + <i>Methanobacterium</i>	0.295 ± 0.028	24.3	0.4	0.4	0.0	9.0	27.0

^aResults obtained after incubation at 62°C.

^bIsobutyrate, isovalerate, and 2-methylbutyrate concentrations did not change after adding serine in the absence or presence of an electron acceptor as compared to the respective control.

methanogenesis [4,11,13,34]. This can be explained by the fact that oxidative deamination of leucine, isoleucine, and valine is endergonic under standard conditions Eqn [see equations (1)Eqn [Eqn [-3]], and the removal of reducing equivalents renders the reactions thermodynamically possible. Therefore, the presence of H_2 oxidizing methanogens is crucial for oxidative deamination of amino acids to occur [11,26]. This concept is strengthened by observations on the proteolytic anaerobic bacterium *Coprothermobacter proteolyticus* [35] (formerly renamed *Thermobacteroides proteolyticus* [23]). First it was thought that it did not use amino acids [23], however, when co-cultured with a hydrogenotrophic methanogen, more acetate, propionate, and isobutyrate were produced from gelatin utilization [12] suggesting that the co-culture probably could oxidize amino acids via interspecies hydrogen transfer. The importance of interspecies hydrogen

transfer in amino acid degrading co-cultures was also demonstrated for hydrogen-oxidizing sulfate-reducers in the presence of sulfate [8]. Smith and Klug [36] concluded that in sediments of Winter Green Lake, a major part of the amino acids were degraded by sulfate-reducing bacteria.

Thermoanaerobacter brockii. was isolated from diverse thermal environments (water, sediment, and decomposing photosynthetic bacteria biomass) in Yellowstone National Park [19], lake sediments from Lake Kivu in East Africa, and oil wells [22]. It is most likely that thiosulfate could be generated chemically in such environments, because of the simultaneous presence of H_2S and O_2 [19,37,38]. Therefore, our results demonstrate that in the presence of thiosulfate, thermophilic thiosulfate-reducers such as *T. brockii* could play a significant role in amino acids degradation in thermal environments known to have a high organic content associated with decomposing biomass [19]. In addition, we found that thiosulfate could be replaced by a hydrogenotrophic methanogen acting as a biological electron acceptor during amino acid oxidation by *T. brockii*. We also observed that the presence of thiosulfate or an hydrogenotroph delayed cell lysis of *T. brockii* [see Figures 1(a) and (b)] grown on the amino acids tested. This phenomenon might be due to ATP generated during amino acid oxidation.

Our results suggest that the ability of microorganisms to use amino acids and/or proteins should be tested in the presence of a H_2 scavenger (methanogen or sulfate-reducing bacterium) or a chemical electron acceptor such as thiosulfate or elemental sulfur to better define their phenotypic characteristics. This is in line with Mead [1] who proposed that amino acid catabolism should be used as a systematic comparative key for *Clostridium* sp. in a way analogous to that commonly used for sugar catabolism. We suggest that such tests should be extended to include the peptide-using thermophiles and/or hyperthermophiles from domains *Archaea* and *Bacteria* [39].

In this work, we have identified the amino acids that are fermented or oxidized to acetate, isobutyrate, isovalerate, and 2-methylbutyrate, but not the amino acid from which traces of propionate are produced. Additionally, the absence of *n*-butyrate as an end-product from amino acid metabolism by *T. brockii* clearly indicates that it cannot couple the oxidation of amino acid to the reduction of exogenous acetate as reported for some other anaerobes [11].

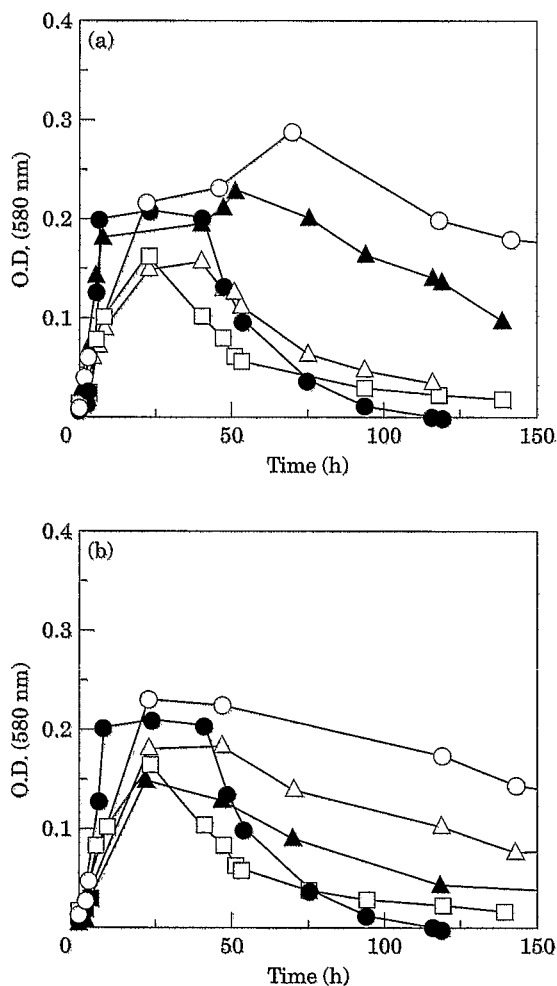


Figure 1. Effect of thiosulfate or *Methanobacterium* sp. as electron acceptors on (a) isoleucine and (b) valine oxidation by *Thermoanaerobacter brockii*. Key: control without amino acid (□); control without amino acid supplemented with 20 mM thiosulfate (●); amino acid (Δ); amino acid supplemented with thiosulfate (▲); amino acid + *Methanobacterium* sp. (○).

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