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Why Does *Clostridium acetireducens* Not Use Interspecies Hydrogen Transfer for Growth on Leucine?

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Abstract. *Clostridium acetireducens* is the first reported anaerobic bacterium that is dependent on acetate as an electron acceptor for growth on branched-chain amino acids and alanine. The fermentation pathway of leucine and its deamination product α-ketoisocaproate were studied in this organism. Addition of *Methanobacterium formicicum* to pure cultures of *C. acetireducens* stimulated the degradation of α-ketoisocaproate but not the degradation of leucine, indicating that the electrons produced during the oxidative deamination of leucine were not transferred to hydrogen. This conclusion is supported by the observed low NAD(P)H ferredoxin reductase activity. Not only acetate but also crotonate proved to be an appropriate electron sink for the regeneration of $NAD(P)^+$ in this bacterium. Interestingly, *C. acetireducens* was shown to form polyhydroxybutyrate during growth on leucine plus acetate.

Because of the ubiquitous occurrence of proteins in almost any ecosystem, the turnover of amino acids is a very important microbiological process. Anaerobic degradation of amino acids usually starts with a deamination [1]. This deamination can be oxidative, reductive, or occur via an α/β-elimination without oxidation/reduction [1, 19]. The Gibbs free energy changes for oxidative deaminations of amino acids are highly endergonic and can occur only if the electrons produced in this step are effectively scavenged [19, 20, 25, 29]. This is known to be possible when the electrons are taken up via interspecies hydrogen transfer by methanogens or sulfate reducers [20, 25], or by another amino acid in the so-called Stickland reaction [23].

Recently, an anaerobic bacterium, *Clostridium acetireducens*, was isolated in our laboratory with yet another mechanism for scavenging electrons produced in the deamination step: this bacterium uses acetate as an electron acceptor and produces butyrate as the reduced end product during the fermentation of leucine, isoleucine, valine, or alanine [21]. *C. acetireducens* is the first example of an organism strictly dependent on acetate for growth on amino acids. Acetate has been reported to be used as an electron acceptor by *C. tyrobutyricum* and *C. butyricum* for complete degradation of mannitol [12, 14] or lactate in the case of *C. tyrobutyricum* [7]. *C. beijerinckii* and *C. kluyveri* need to reduce acetate to butyrate for growth on lactate and ethanol, respectively [3, 4, 15]. The only bacterium shown to reduce acetate to butyrate during growth on amino acids (isoleucine) is *Eubacterium limosum* [11], but the amounts of acetate reduced were so low that a dependence on acetate as an electron acceptor can be ruled out.

In this paper, we present preliminary evidence that *C. acetireducens* cannot use interspecies hydrogen transfer in co-culture with *Methanobacterium formicicum* for growth on leucine, because of its inability to transfer electrons from NAD(P)H to hydrogen.

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Materials and Methods

Source of organisms. *Clostridium acetireducens* was isolated from an acidic bioreactor of the purification plant of the AVEBE potato-starch factory, De Krim, The Netherlands, as described earlier [21] and has been deposited under No. 10703 of the DSMZ, Braunschweig, Germany. *Methanobacterium formicicum* was kindly provided by A.J.M. Stams.

Media and cultivation methods. Medium composition and preparation for growth of *C. acetireducens* have been described by Örlygsson et al. [21] and Nanninga and Gottschal [18]. The co-culture of *C. acetireducens* and *M. formicicum* was done as follows: *M. formicicum* was pregrown in the presence of the substrates to be studied in the co-culture and with H_2/CO_2 (80/20% [vol/vol]) as the gas phase. When visible growth had occurred (3–5 days), the gas phase was exchanged for N_2/CO_2 (80/20% [vol/vol]) and the culture was inoculated with *C. acetireducens* (5% [vol/vol]). Growth experiments were done in screw-cap tubes (15 ml) and bottles (120 and 250 ml) sealed with butyl rubber stoppers and aluminum crimp seals.

Chemostat culture. The working volume of the bioreactor used for chemostat cultivation was 535 ml. The chemostat was stirred at 250 rpm, the temperature was maintained at 37°C, and the pH at 7.3. The composition of the medium was the same as used in batch cultures for growth on leucine (20 mM) plus acetate (30 mM). Both the chemostat and the reservoir medium were kept under N_2/CO_2 (80/20% [vol/vol]) atmosphere. The inoculum size was 5% of the culture volume. The culture was grown batchwise during 17–20 h before the medium flow was started at a dilution rate of 0.05 h^{-1}.

Preparation of cell-free extract. Anaerobic conditions were maintained throughout the entire procedure by performing all steps under a $N_2/CO_2/H_2$ (80/10/10% [vol/vol/vol]) gas phase. Ninety-milliliter samples were removed from the chemostat culture after a steady state was obtained (≥5 volume changes) and centrifuged (10,000 rpm for 30 min, at 4°C). Cell pellets were resuspended in 3 ml of 100 mM oxygen-free (flushed with N_2) Tris/HCl buffer (pH 7.6) containing 2 mM of dithiothreitol. Extracts were prepared anaerobically by passing the cell suspension through a French pressure cell. Cell debris was removed by centrifugation (19,000 rpm for 30 min, at 4°C), and the resulting cell-free extract was stored anaerobically at −80°C. Protein content of cell-free extracts was estimated with Coomassie Brilliant Blue, with bovine serum albumin as a standard.

Enzyme assays. Enzyme activities were determined at 37°C in 1.5-ml quartz cuvettes flushed with N_2 and sealed with a butyl rubber stopper. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 µmol of substrate per minute. The following assays were adopted from Vasconcelos et al. [28]: NAD(P)H ferredoxin reductase (with ferredoxin from red marine algae), hydrogenase in the direction of hydrogen consumption, phosphotransacetylase, phosphotransbutyrylase, acetate kinase, butyrate kinase, acetoacetyl-CoA:acetate CoA-transferase. The assay for the α-ketoisocaproate ferredoxin oxidoreductase was performed according to the procedure described by Lovitt et al. [16] to assay the pyruvate ferredoxin oxidoreductase except that 5 mM of pyruvate was replaced by 5 mM of α-ketoisocaproate. The ferredoxin $NAD(P)^+$ reductase was assayed by the method of Petitdemange et al. [22], with pyruvate as the reductant of ferredoxin. The hydrogenase activity in the direction of hydrogen formation was measured by the gas chromatography method of Jungermann et al. [13], modified as follows: the assay was carried out at 37°C in 10-ml flasks sealed with a butyl rubber stopper and an aluminum crimp seal, and flushed with nitrogen. The assay mixture contained 50 mM Tris/HCl (pH 8), 60 mM sodium dithionite, 2 mM methylviologen in a total volume of 2 ml. The determination of the butyryl-CoA:acetate CoA-transferase activity was performed according to the method of Sramek and Frerman [24]. For the determination of the isovaleryl-CoA:acetate CoA-transferase activity, 2 mM of butyryl-CoA was replaced by 2 mM of isovaleryl-CoA.

Analytical methods. Supernatants were used from centrifuged (10 min, 10,000 rpm) cell suspensions for the analysis of various end products. For the analysis of volatile fatty acids, the supernatant was acidified by adding 50 µl of 10% phosphoric acid to a 500-µl sample. Valeric acid was used as an internal standard. Acidified samples were injected in a Varian 3600 gas chromatograph equipped with an Alltech ECONO-cap column (30 m × 0.53 mm). Nitrogen was used as carrier gas at a flow rate of 12 ml min^{-1}, and 1-µl samples were injected with an automatic liquid sampler (Varian 8100). The oven temperature was programmed to remain at 110°C for 2 min and then to increase linearly to 120°C at a rate of 10°C per min, and finally to remain at 120°C for 1 min. Injector and detector (FID) temperatures were 250°C. Polyhydroxybutyrate (PHB) was extracted from cells according to Braunegg et al. [5], and 2-µl samples were injected on a Varian 3600 gas chromatograph. The same conditions were used as described above. PHB was subsequently quantified as 3-hydroxybutyrate methyl ester. The amounts of cell carbon, cell densities, hydrogen, and methane were determined as previously described [21].

Results

Mixed culture experiments. To investigate the influence of interspecies hydrogen transfer on the metabolism of *C. acetireducens*, we co-cultured the organism with *M. formicicum* during growth on leucine and α-ketoisocaproate (α-KIC) in the absence and in the presence of acetate (Table 1). In the absence of acetate, incubation on leucine plus methanogens did not result in detectable growth of *C. acetireducens*. However, growth on α-KIC without acetate was positively affected by the removal of hydrogen as 8.1 mM additional isovalerate was formed and hence more α-KIC was used in the presence of methanogens. In the presence of acetate, the degradation of leucine and α-KIC by *C. acetireducens* was not significantly affected by the presence of methanogens. Both substrates were completely (or almost completely) degraded, with the main difference that some reducing equivalents were now transferred via hydrogen to methane. The amount of methane from leucine + acetate was almost twice that from α-KIC + acetate, as expected on the basis of the higher hydrogen production from leucine + acetate.

Crotonate utilization. Crotonate could be used both as the sole carbon and energy source and as a co-substrate during leucine fermentation (Table 2). In both cases crotonate was fermented to acetate and butyrate but in different proportions. On 30 mM crotonate, the acetate/butyrate ratio reached the value of 1.5, while in presence of leucine no more acetate was produced. The acetate/butyrate ratio of 1.85 during growth on 60 mM crotonate dropped to 0.5 in the presence of leucine.

Enzyme activities. To further clarify the metabolism of *C. acetireducens*, we assayed some enzymes involved in the electron flow and in the conversion of acetate to butyrate in *C. acetireducens* cells sampled from a chemostat culture on leucine plus acetate (Table 3, Fig. 1).

Table 1. Mixed cultures of *C. acetireducens* with *Methanobacterium formicicum* (M) grown on leucine and α-ketoisocaproate in the presence and in the absence of acetate

Substrate	Residual[a] substrate (mM)	Biomass[b] (mgC l^{-1})	Concentration (mM)					% Recovery	
			Acetate[c]	Butyrate	Isovalerate	Hydrogen[d]	Methane[d]	Carbon[e]	Electrons
Leucine (20 mM)	19.5	3	0	0	0.2	<0.1	–[f]	–	–
Leucine (20 mM) + M	18.7	5	0	0	1.4	<0.1	0.10	113	100
Leucine (20 mM) + acetate (30 mM)	1.4	100	4.3	11.0	16.2	5.3	–	92	90
Leucine (20 mM) + acetate (30 mM) + M	1.2	104	4.0	10.5	16.1	<0.1	1.43	90	88
α-Ketoisocaproate (20 mM)	13.9	37	0	0	5.4	2.2	–	97	92
α-Ketoisocaproate (20 mM) + M	6.2	87	0	0	13.5	<0.1	0.45	107	99
α-Ketoisocaproate (20 mM) + acetate (30 mM)	0	120	11.8	7.0	20.8	2.5	–	103	102
α-Ketoisocaproate (20 mM) + acetate (30 mM) + M	0	126	14.7	4.6	18.6	<0.1	0.84	94	93

The pure culture results were included for comparison. In all incubation experiments, the biomass and product concentrations were corrected for the presence of 2 g L^{-1} of yeast extract in the medium. Samples were taken after incubation for 15 days, and the values are means of two parallel bottles.
[a] Concentration of leucine or α-ketoisocaproate left in the experimental bottles at the end of the fermentation.
[b] Total organic carbon concentration in pellets of the cultures.
[c] Acetate concentration left in the experimental bottles.
[d] The amount of hydrogen and methane detected in the gas phase is expressed in millimoles per liter of culture liquid.
[e] In presence of leucine or α-ketoisocaproate, the percentage of carbon recovery was calculated assuming that one mole of CO_2 was produced per mole of isovalerate produced.
[f] –: not determined.

α-Ketoisocaproate ferredoxin oxidoreductase activity was detected in cells grown on leucine + acetate. Enzyme activities for hydrogen consumption and hydrogen formation were detected. Very low activity was found for the reduction of ferredoxin by NAD(P)H, whereas the reverse activity from reduced ferredoxin to $NAD(P)^+$ was much higher. No higher NAD(P)H ferredoxin reductase activity was obtained by increasing the concentration of its activator acetyl-CoA, or by making changes to the concentrations of any of the other components of the assay. The acetate kinase and phosphotransacetylase (both measured in the direction of acetyl phosphate formation) had a similar activity. CoA-transferase activity was tested with three different acyl-CoA species as substrate: butyryl-CoA, acetoacetyl-CoA, and isovaleryl-CoA. No CoA-transfer was found between butyryl-CoA and acetate, but significant transferase activity was observed when acetoacetyl-CoA and isovaleryl-CoA were used as CoA-donor to activate acetate. Other enzymes involved in the conversion of acetyl-CoA to butyrate, such as β-hydroxybutyryl-CoA dehydrogenase, butyrate kinase, and phosphotransbutyrylase, were also detected in *C. acetireducens*.

PHB production. Electron microscopical observations have indicated that cells of *C. acetireducens* grown on leucine plus acetate contained large amounts of intracellular granules [21]. Chemical analysis by gas chromatography indicated that these granules consisted of polyhydroxybutyrate (PHB).

Discussion

The mixed-culture experiments of *C. acetireducens* with *M. formicicum* showed that growth on leucine alone or on leucine + acetate was not improved by the presence of methanogens. This confirms the inability of *C. acetireducens* to grow on leucine via interspecies hydrogen transfer [21]. In contrast, growth on α-KIC was significantly increased by the presence of *M. formicicum*, and methane was formed instead of hydrogen. This indicates that the formation of methane from reducing equivalents generated during the oxidative decarboxylation of α-KIC is quite possible, probably involving α-ketoisocaproate ferredoxin oxidoreductase and hydrogenase activities of *C. acetireducens*. A very low activity was found for the NAD(P)H ferredoxin reductase. With the same assay, a

Table 2. Fermentation patterns of *C. acetireducens* during growth on crotonate or leucine + crotonate at different concentrations

Substrate	Biomass[a] (mgC l^{-1})	Concentration (mM)				% Recovery	
		Acetate	Butyrate	Isovalerate	Hydrogen[b]	Carbon[c]	Electrons
Crotonate (30 mM)	328	24.6	15.5	–[d]	3.9	115	115
Crotonate (60 mM)	682	48.2	26.1	–	3.5	107	106
Leucine (20 mM) + crotonate (30 mM)	488	nd[e]	27.0	18.4	6.6	108	105
Leucine (20 mM) + crotonate (60 mM)	572	27.2	49.8	17.6	7.4	113	112

In all cultures, the biomass and product concentrations were corrected for the presence of 2 g L^{-1} of yeast extract in the medium. Samples were taken after incubation for 10 days, and the values are means of two parallel bottles. The substrates were degraded completely in all cases.
[a] Total organic carbon concentration in pellets of the cultures.
[b] The amount of hydrogen detected in the gas phase is expressed in millimoles per liter of culture liquid.
[c] In presence of leucine, the percentage of carbon recovery was calculated assuming that one mole of CO_2 was produced per mole of isovalerate produced.
[d] –: not determined.
[e] nd: undetectable.

very high activity (0.17 μmol min^{-1} mg^{-1}) was observed in a cell-free extract of *Clostridium acetobutylicum*, which was used as a positive control after growth on glucose. The low level of activity found for NAD(P)H ferredoxin reductases indicates that the electron transfer from reduced nucleotides to oxidized ferredoxin is not likely in vivo. This means that reducing equivalents in the form of reduced nucleotides, generated during the deamination step of leucine, are apparently not oxidized via hydrogen formation (with ferredoxin used as an electron carrier). This may well explain the inability of *C. acetireducens* to grow on leucine via interspecies hydrogen transfer. It represents an even more strict dependence on acetate as an electron sink, as already observed by Heyndrickx et al. [12]. These latter authors proposed that NADH ferredoxin oxidoreductase activity of *C. butyricum* formed a "bottleneck" in the electron flow of the fermentation of mannitol, which was circumvented by the reduction of acetate to butyrate. Similarly, Britz and Wilkinson [6] showed that the oxidizing agent methylene blue enhanced the capacity of *Clostridium bifermentans* extract to deaminate and decarboxylate leucine.

We have measured the in vitro activities of enzymes involved in the reduction of acetate to butyrate in a chemostat culture of *C. acetireducens* grown on leucine plus acetate. Conversion of acetate into butyrate requires activation of acetate to acetyl-CoA as the first step. The results from these enzyme measurements demonstrated the presence in *C. acetireducens* of at least two possible acetate activation pathways. First, acetate could be converted to acetyl-CoA in two steps via an acetate kinase and a phosphotransacetylase. Second, acetate could be activated by a CoA-transferase with acetoacetyl-CoA or isovaleryl-CoA as substrate, but not butyryl-CoA. It is not possible on the basis of these enzyme measurements to decide which of the two possible routes is more important in vivo. The subsequent reductive step is catalyzed by NADH-dependent β-hydroxybutyryl-CoA dehydrogenase. Butyrate is then formed via a phosphotransbutyrylase and a butyrate kinase.

Table 3. In vitro specific activities of enzymes involved in leucine and acetate metabolism in cells of *C. acetireducens* grown in chemostat culture on leucine (20 mM) plus acetate (30 mM)

Enzyme[a]	Activity in μmol min^{-1} (mg protein)$^{-1}$
1. α-Ketoisocaproate ferredoxin oxidoreductase	0.51 ± 0.14
2. Ferredoxin NAD(P)H oxidoreductase:	
ferredoxin NAD^+ reductase[b]	0.099 ± 0.015
ferredoxin $NADP^+$ reductase[b]	0.070 ± 0.014
NADH ferredoxin reductase	0.007 ± 0.002
NADPH ferredoxin reductase	0.003 ± 0.001
3. Hydrogenase:	
hydrogen consumption	0.46 ± 0.02
hydrogen formation	0.14 ± 0.02
4. Acetate kinase	0.44 ± 0.07
5. Phosphotransacetylase	0.40 ± 0.10
6. CoA-transferase:	
butyryl-CoA:acetate CoA-transferase	nd[c]
acetoacetyl-CoA:acetate CoA-transferase	0.47 ± 0.18
isovaleryl-CoA:acetate CoA-transferase	5.22 ± 1.80
7. β-Hydroxybutyryl-CoA dehydrogenase:	
NADH	1.04 ± 0.26
NADPH	0.49 ± 0.13
8. Phosphotransbutyrylase	1.34 ± 0.29
9. Butyrate kinase	2.37 ± 0.16

The values represent the mean ± the standard deviation (n = 4).
[a] Numbers refer to the reactions shown in Fig. 1.
[b] The pyruvate ferredoxin oxidoreductase present in the cell-free extract was used as coupling enzyme in the assay. Its in vitro activity was 1.36 ± 0.08 μmol min^{-1} (mg protein)$^{-1}$, with the assay described by Lovitt et al. [16].
[c] nd: undetectable.

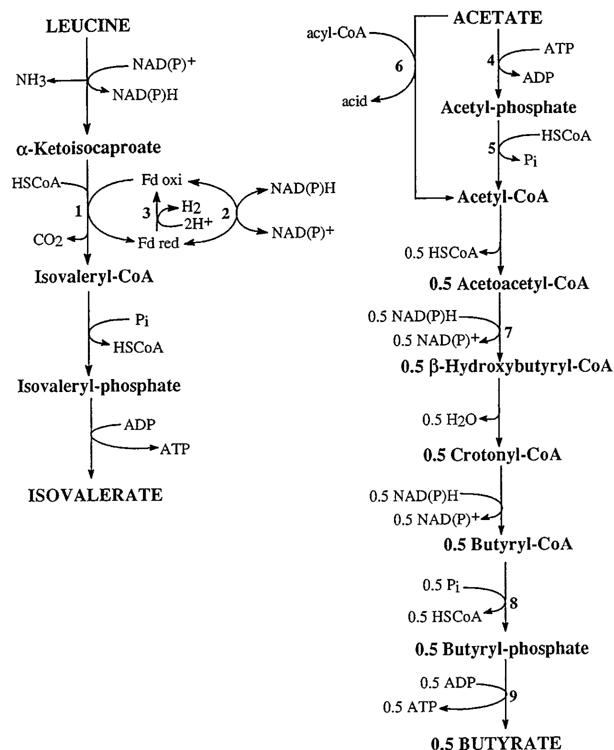

Fig. 1. Schematic representation of the pathways for leucine and acetate metabolism on the basis of previously published routes [1]. The numbers refer to the enzymes with their activities shown in Table 3. Fd oxi: oxidized ferredoxin; Fd red: reduced ferredoxin.

We have also shown that *C. acetireducens* is able to use crotonate as its sole source of carbon and energy, as reported earlier for several *Clostridium* species [2, 8, 9, 27]. In the presence of leucine, the increased formation of butyrate relative to acetate indicates that crotonate was used as an electron acceptor. For *Syntrophomonas wolfei*, it is known that crotonate is initially activated to its CoA-derivative, crotonyl-CoA [17]. It is possible to suppose that the CoA-transferase system, detected in *C. acetireducens* grown on leucine plus acetate, is involved in crotonate activation. However, this requires further enzyme studies, since CoA-transferases seem to be quite specific enzymes [2] and since in *S. wolfei* growth on crotonate requires a CoA-transferase activity with altered substrate specificity or the synthesis of a new enzyme specific for crotonate [17]. Furthermore, a comparison of the phosphotransacetylase and acetate kinase activities found in *C. acetireducens* with those reported for other crotonate-fermenting anaerobes shows a great variability in the level of these two enzymes. A very high phosphotransacetylase activity (143 μmol min^{-1} mg^{-1}) was found in *S. wolfei* [17], while low activities (between 0.4 and 1.4 μmol min^{-1} mg^{-1}) were measured in *Ilyobacter polytropus* [26], *Clostridium homopropionicum* [9], and *C. acetireducens* [this study]. The acetate kinase activities found in *S. wolfei* and *I. polytropus* [17, 26] were seven times higher than those measured in the two clostridia, *C. homopropionicum* and *C. acetireducens* [9, this study]. This indicates that the phosphotransacetylase and acetate kinase activities are strongly dependent on the organism, on the substrate used for growth, and possibly, in the case of the phosphotransacetylase, on the direction (physiological or reverse) in which the enzyme was assayed.

The observed accumulation of PHB in cells of *C. acetireducens* grown on leucine plus acetate raises the question as to whether and how PHB can be utilized as a carbon and energy source by this strictly anaerobic organism. *Clostridium botulinum* is the only other *Clostridium* known to produce PHB [10]. In *C. botulinum*, PHB granules are utilized as carbon and energy sources for spore maturation, but the metabolic routes involved are not known.

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