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Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA

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Abstract

Fermentation balances and growth yields were determined with various bacteria fermenting lactate to acetate plus propionate either via methylmalonyl CoA or via acrylyl CoA. All strains fermented lactate to acetate plus propionate at approximately a 1:2 ratio. Growth yields of *Propionibacterium freudenreichii* were more than twice as high as those of *Clostridium homopropionicum* or *Veillonella parvula*. Hydrogen was formed as a side product to a significant extent only by *V. parvula* and *Pelobacter propionicus*; the latter formed hydrogen preferentially when using ethanol as substrate. Acrylyl CoA reductase of *C. homopropionicum* and *Clostridium neopropionicum* was found nearly exclusively in the cytoplasm thus confirming that this reduction step is unlikely to be involved in energy conservation. *C. homopropionicum* exhibited higher K_S and higher μ_{max} values, as well as higher specific substrate turnover rates than *P. freudenreichii*. The results allow us to conclude that *C. homopropionicum* using the acrylyl CoA pathway with low growth yield obtains its specific competitive advantage compared to *P. freudenreichii* not through higher substrate affinity or metabolic shift toward enhanced acetate plus hydrogen formation but through faster specific substrate turnover.

Keywords: Propionate fermentation; Methylmalonyl-CoA pathway; Acrylyl-CoA pathway; Energetics

1. Introduction

Lactate is fermented to acetate and propionate by various bacteria including *Propionibacterium* spp. and many others. The overall fermentation according to:

$$3\ CH_3CHOHCOO \rightarrow CH_3COO + 2\ CH_3CH_2COO + CO_2 + H_2O \quad (1)$$

$$\Delta G^{o\prime} = 170\ \text{kJ per 3 mol lactate}$$

yields sufficient energy to allow synthesis of at least 2 mol ATP per 3 mol lactate, assuming that an amount of at least +70 kJ is needed for synthesis of 1 mol ATP under physiological conditions [1,2]. Bacteria forming propionate via the methylmalonyl-CoA pathway, e.g. *Propionibacterium freudenreichii*, synthesize 1 ATP by substrate-level phosphorylation via phosphotransacetylase and acetate kinase, and further $2 \times 2/3$ ATP by electron transport phosphorylation in fumarate reduction to succinate [3,4]. In contrast, bacteria using the acrylyl-CoA pathway have not been reported to be able to conserve metabolic energy in the reductive branch of their fermentation [5], thus leaving them with only 1 mol ATP formed per 3 mol lactate fermented. *Veillonella parvula* uses the methylmalonyl-CoA pathway including fumarate reductase-dependent electron transport phosphorylation. However, it carboxylates pyruvate to oxaloacetate by an ATP-consuming step, the energy of which is recovered only in part by a sodium ion-translocating methylmalonyl-CoA decarboxylase [6 8]. Thus, the energy yield for *V. parvula* during lactate fermentation should be substantially lower than that of *P. freudenreichii*.

The expected differences in energy yields of these fermentations raised the question what the strategic advantage of lower energy conservation compared to highly efficient energy exploitation might be. Strategies could aim at higher substrate affinity, higher specific substrate turnover rates, or higher metabolic flexibility, e.g. by shifting the

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fermentation towards higher acetate formation with simultaneous interspecies hydrogen transfer. We therefore examined the energy metabolism and growth yields of bacteria using the various fermentation pathways under comparable conditions, checked for possible energy conservation in acrylyl-CoA reduction, and also examined the fermentation stoichiometries under various growth conditions.

2. Materials and methods

2.1. Strains used

Clostridium homopropionicum (DSM 5847^T), *Clostridium neopropionicum* (DSM 3847^T), *P. freudenreichii* (DSM 20271^T), *V. parvula* (DSM 2008^T), *Pelobacter propionicus* (DSM 2379^T) and *Methanospirillum hungatei* (DSM 864^T) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany.

2.2. Cultivation

All strains were grown in bicarbonate-buffered cysteine- or sulfide-reduced mineral salts medium as described earlier [9,10]. Growth experiments were carried out in 26-ml Hungate tubes sealed with butyl rubber stoppers and filled with 13 ml culture medium; headspaces were gassed with N_2/CO_2 (80/20%). Growth was followed by turbidity measurement with a Spectronic 70 spectrophotometer at 540 nm wavelength (Bausch and Lomb, Rochester, MN, USA). Growth yield determinations were calibrated by gravimetric determination of dry cell mass grown in 1-l cultures. Larger amounts of cells were grown either in 10-l bottles or in serum bottles of various sizes, sealed with butyl rubber stoppers. For cultivation in continuous culture, 300- or 500-ml vessels were used with no headspace, sealed with butyl rubber septa.

2.3. Kinetic studies

Half-saturation constants for substrate uptake were determined in continuous culture or in static assays with dense cell suspensions. Substrate depletion curves of cell suspensions were fitted with a non-linear regression analysis based on a three-parameter version of the Michaelis Menton equation (Origin, MicroCal Software, Northampton, MA, USA).

2.4. Preparation of cell-free extracts

Cells were harvested in the late exponential growth phase and washed with oxygen-free 50 mM potassium phosphate buffer, pH 7.0. Cells were broken by repeated French press treatment anoxically under N_2, and unbroken cell debris was removed by centrifugation at $10\,000 \times g$ for 10 min. Cytoplasmic fraction and membranes were separated by centrifugation at $100\,000 \times g$ for 30 min.

2.5. Cytochromes

Cytochromes were determined in cell-free extracts and in membrane and cytoplasmic fractions via redox difference spectra (dithionite-reduced minus air-oxidized) taken with a double-beam spectrophotometer (Uvikon, Kontron, Zürich, Switzerland). Cytochromes were identified by their absorption peaks [11]; cytochrome *b* was quantified using an extinction coefficient of 17.5 cm^2 μmol^{-1} for the α-band [12].

Table 1
Fermentation stoichiometries and growth parameters of bacteria fermenting lactate to acetate and propionate

Strain	Incubation	Growth condition	Lactate consumed (mM)	Products formed (mM)				Ac.:prop. ratio	p_{H2}^{max} (Pa)	Y_S (g mol^{-1})
				acetate	propionate	hydrogen	methane			
C. homopropionicum	N_2/CO_2	batch	16.7	7.3	14.3	0.5		1:1.95	530	2.6
	N_2/CO_2	continuous	10.0	3.0	5.9			1:1.99		1.5
	$N_2/CO_2/H_2$	batch	23.5	7.2	14.2			1:1.98		2.4
	M. hungatei	batch	21.6	6.9	14.0	< 0.1	0.4	1:2.03		3.0
P. freudenreichii	N_2/CO_2	batch	8.7	6.4	13.2	0		1:2.06		6.0
	N_2/CO_2	continuous	10.2	3.3	6.9			1:2.09		5.5
	$N_2/CO_2/H_2$	batch	17.2	6.5	13.6			1:2.09		6.3
V. parvula	N_2/CO_2	batch	23.1	6.5	12.2	1.0		1:1.87	2500	2.2
	N_2/CO_2	continuous	16.3	5.3	10.0			1:1.88		2.4
	$N_2/CO_2/H_2$	batch	20.0	8.1	14.5			1:1.79		2.7
	M. hungatei	batch	21.5	8.2	12.4	< 0.1	1.5	1:1.51		3.0
P. propionicus	N_2/CO_2	batch	5.6	1.9	3.54	< 0.1		1:1.86	150	3.82

Batch culture experiments were carried out under three different incubation conditions: N_2/CO_2 atmosphere, N_2/CO_2 atmosphere plus 0.5 bar hydrogen, N_2/CO_2 atmosphere and excess *M. hungatei* (optical densities of 0.15 0.25). All data are average results of five growth experiments corrected against control sets without substrate addition.

2.6. Enzyme assays

Enzyme assays were carried out anoxically at 25°C in 1.5-ml glass or quartz cuvettes with 1-cm light path sealed with rubber stoppers. All reagents were degassed and flushed twice with N_2. Absorbance was followed with a spectrophotometer (100-40, Hitachi, Tokyo). Fumarate reductase was measured according to [13], with 5 mM $MgCl_2$ instead of 10 mM $MgSO_4$ in the buffer. Acyl-CoA dehydrogenases were measured with a 2,6-dichlorophenolindophenol-dependent assay [14]. Acrylyl-CoA reduction was quantified with reduced benzyl viologen as electron donor, 5 mM acrylate as substrate, and 0.15 mM propionyl-CoA for acrylate activation via CoA-transferase.

2.7. Chemical analyses

Alcohols and fatty acids were determined by ion exchange liquid chromatography [15] with optical refraction detection (column: ORH-80, Interaction Chromatography, San Jose, CA, USA; detector: ERC-7512, Sykam, Gauting, Germany). Hydrogen was measured by gas chromatography (Carbosieve SII, Sulpeco, Sulzbach, Germany) with heat conductivity detection (HWD 430, Carlo Erba, Milan, Italy) or by a mercury reduction detector (RGD2, Trace Analytical, Menlo Park, CA, USA). Protein was determined with a protein test kit (BioRad) modified after [16].

2.8. Chemicals

Chemicals used were of analytical grade, and were obtained from Fluka (Buchs, Switzerland), Riedel-de Haën (Seelze, Germany) and Merck (Darmstadt, Germany). Acyl-CoA esters were purchased from Sigma (München, Germany).

3. Results

3.1. Fermentation stoichiometries and growth yields

Fermentation stoichiometries and growth yields were determined in static and in continuous cultures of *C. homopropionicum*, *P. freudenreichii*, *V. parvula*, and *P. propionicus*, with lactate as substrate. As shown in Table 1, all strains fermented lactate to acetate and propionate, nearly exactly at a 1:2 ratio. The presence of hydrogen in the gas phase had no influence on the fermentation pattern. Only *C. homopropionicum* and *V. parvula* produced small amounts of hydrogen as a side product; addition of *M. hungatei* as a hydrogen scavenger did not cause a significant shift of the fermentation balance towards increased acetate formation. As expected, molar growth yields of *P. freudenreichii* were more than twice as high as those of *C. homopropionicum* or *V. parvula*.

3.2. Localization of acrylyl-CoA reductase activity

Acrylyl-CoA reductase activity was measured in a coupled test with acrylate and propionyl-CoA since efforts to chemically synthesize acrylyl-CoA failed. In cell-free extracts of *C. homopropionicum*, acrylyl-CoA reduction with reduced benzyl viologen was measured at 2.5 3.2 μmol min ^{1} (mg protein) ^{1}, which was equivalent to the physiological activity of growing cells (2.3 3.0 μmol min ^{1} (mg protein) ^{1}). This activity was nearly exclusively ($>90\%$) localized in the cytoplasmic fraction. In the reverse direction with 2,6-dichlorophenol indophenol as acceptor, an acyl-CoA dehydrogenase was detected which exhibited far lower activities (0.02 0.09 μmol min ^{1} (mg protein) ^{1}), both with propionyl-CoA or butyryl-CoA. This activity was not membrane-bound either. Similar results were obtained with extracts of *C. neopropionicum* (not shown). In extracts of *P. freudenreichii*, *V. parvula*, and *P. propionicus*, no acrylyl-CoA reductase activity could be found.

3.3. Ethanol fermentation by P. propionicus

P. propionicus can also use ethanol as substrate. Acetate and propionate were formed again at a 1:2 ratio, but the molar growth yield with ethanol was less than half that obtained with lactate (Table 2). Ethanol fermentation in the presence of hydrogen led to significant formation of propanol as a coproduct, probably through exchange between acetyl-CoA and propionyl-CoA. Moreover, ethanol

Table 2
Fermentation stoichiometries and growth parameters of *P. propionicus* growing with ethanol

Incubation	Substrate consumed (mM)	Products formed (mM)					Ac:prop. ratio	p_{H2}^{max} (Pa)	μ_{max} (h ^{1})	Y_S (g mol ^{1})
		acetate	propionate	hydrogen	methane	other				
N_2/CO_2	21.8	8.4	14.5	0.7			1:1.73	3300	0.10	1.6
$N_2/CO_2/H_2$	22.2	8.3	9.7			3.1 (propanol)	1:1.17		0.09	1.4
M. hungatei	22.9	12.7	8.6	<0.1	5.4		1:0.68		0.12	1.7

Experiments were carried out under three different incubation conditions, in the absence or presence of *M. hungatei* (optical densities of 0.15 to 0.25). All data are average results of five growth experiments corrected against control sets without substrate addition.

Table 3
Summary of energetic and kinetic data of propionate fermentation by different bacteria

Organism	Pathway	μ_{max} (h^{-1})	Y_S (g mol^{-1})	q_{max} (lactate) (μmol h^{-1} g^{-1})	ATP/lactate (mol mol^{-1})	q_{max} (ATP) (μmol h^{-1} g^{-1})	K_S (μM)	$a_0 = q_{max} K_S^{-1}$ (l h^{-1} g^{-1})
C. homopropionicum	Acrylyl-CoA	0.33	2.6	127	0.33	42	560 ± 210	0.113
P. freudenreichii	MeMal-CoA	0.12	6.0	20	0.78	15.6	140 ± 30	0.118
V. parvula	MeMal-CoA	0.20	2.2	91	0.33	30	290 ± 180	0.280
P. propionicus	MeMal-CoA	0.03	3.82	7.9	0.78	6.2	N.D.	N.D.

MeMalCoA = methylmalonyl-CoA.

fermentation released substantially more hydrogen than lactate fermentation. In the presence of *M. hungatei* as a hydrogen scavenger, ethanol fermentation was shifted substantially towards higher acetate formation (Table 2).

Despite the observed differences in growth yields on lactate versus ethanol, no differences could be detected after growth with either substrate with respect to fumarate reductase activity (0.131 ± 0.08 μmol min^{-1} (mg membrane protein) $^{-1}$) or the cytochrome *b* content (0.69 ± 0.02 μmol (mg membrane protein) $^{-1}$).

3.4. Substrate uptake kinetics

Saturation constants (K_S) were determined with lactate as substrate, either in dense suspensions of statically grown cells or in continuous cultures. *P. freudenreichii* exhibited the highest affinity, i.e. the lowest K_S for lactate, followed by *V. parvula*, and *C. homopropionicum* (Table 3). Substrate depletion kinetics with statically grown cells and K_S values determined in continuous cultures at half-maximal dilution rates did not differ systematically (not shown). On the other hand, the specific lactate comsumption rate, q_{max}, was highest with *V. parvula*, followed by *C. homopropionicum*, and was substantially lower with *P. freudenreichii* and *P. propionicus*. Apparently, the organisms conserving less energy per reaction compensated this disadvantage by higher growth rates and, as a consequence of their low growth yields, even higher substrate consumption rates.

A similar situation is observed with *P. propionicus* growing either with lactate or with ethanol. Growth with ethanol yielded substantially less cell mass than growth with lactate, but ethanol uptake was more than four times faster (36 73 μmol h^{-1} g^{1}) than lactate uptake (7.9 μmol h^{-1} g^{1}; Table 3), thus compensating for the lower energy supply rate provided with this energetically less valuable substrate.

4. Discussion

This study documents that energy conservation during lactate fermentation to acetate plus propionate under directly comparable conditions differs substantially between bacteria using the methylmalonyl-CoA pathway and those using the acrylyl-CoA pathway. As expected, proton translocation during fumarate reduction in the methylmalonyl-CoA pathway adds substantially to the energy yield whereas there was no indication of electron transport phosphorylation in acrylyl-CoA reduction. Acrylyl-CoA reductase of *C. homopropionicum* and *C. neopropionicum* was found nearly entirely in the cytoplasmic cell fraction, thus confirming that this enzyme is unlikely to be involved in ion translocation across the cytoplasmic membrane. In this respect, acrylyl-CoA reduction resembles crotonyl-CoA reduction in butyric acid-forming clostridia [1]. The redox potential of the crotonyl-CoA/butyryl-CoA couple at pH 7.0 was reported to be 125 mV [17]; a more recent publication gives a more positive value, 10 mV [18]. In the same publication, the redox potential of the acrylyl-CoA/propionyl-CoA couple was determined to be substantially more positive, i.e. +69 mV. Although acrylyl-CoA reduction could therefore be coupled to substantial ATP formation it appears that the free energy available in this reaction is released as heat and does not contribute to ATP synthesis. The cell yields obtained (on average 5.9 g per mol lactate with *P. freudenreichii*, and 2.4 g per mol lactate with *C. homopropionicum*) allow calculation of an Y_{ATP} value of 7.2 7.7 g per mol ATP, which is to be expected for growth in a mineral medium with one simple carbon source [19]. The ratio of both values is 2.45 and confirms nearly exactly the assumption that $2 \times 2/3$ ATP is synthesized in fumarate reduction [3].

The obviously lower ATP yield of *C. homopropionicum* compared to *P. freudenreichii* could be compensated by different metabolic strategies. *P. freudenreichii* seems keyed to maximum energy yield during growth in pure culture, which may be of advantage in the rather rich environment it is known to live in. *C. homopropionicum*, on the other hand, might prefer to cooperate with partner organisms, e.g. hydrogen-scavenging methanogens, similar to the butyrate-forming clostridia [20], and increase its ATP yield by a shift to nearly exclusive acetate formation. Our results here document that the strains we tested do not choose this alternative. Hydrogen formation was observed only to a small extent, and cocultivation with a hydrogen-scavenging methanogen did not shift the fermentation stoichiometry significantly.

A disadvantage in energy conservation could also be compensated for by higher substrate affinity to secure en-

ergy supply under conditions of substrate limitation that are typical of natural environments. Contrary to our expectation, *C. homopropionicum* exhibited substantially higher half-saturation constants (K_S) than *P. freudenreichii*. However, *C. homopropionicum* grew at considerably higher rates and, due to its lower growth yield, reached far higher maximal substrate turnover rates (q_{max}) than *P. freudenreichii*. According to Button [21,22] it is not the half-saturation constant K_S itself but the initial slope of the substrate saturation curve that determines the outcome of competition between two organisms with different substrate uptake kinetics. This initial slope (a_0) can be calculated from q_{max} and K_S, and comes out to nearly equal values for *C. homopropionicum* and *P. freudenreichii* (Table 3). Obviously, *C. homopropionicum* compensates its high half-saturation constant by a very high substrate turnover rate.

A different situation occurs with *V. parvula*. This bacterium carboxylates pyruvate to oxaloacetate not by a transcarboxylase reaction with methylmalonyl-CoA as carboxylic group donor, but by an ATP-consuming pyruvate carboxylase [7]. Decarboxylation of methylmalonyl-CoA to propionyl-CoA is coupled to the establishment of a transmembrane sodium ion gradient [23,24] which can conserve the equivalent of 1/3 ATP unit per reaction [25]. If fumarate reduction in this metabolism is coupled to synthesis of 2/3 ATP as assumed above, both reactions add up to cover just the ATP expenditure of pyruvate carboxylation, leaving a balance of 1 ATP per 3 lactate for the lactate fermentation by *V. parvula*, equivalent to the situation with *C. homopropionicum*. The yield data obtained confirm exactly this hypothesis, with nearly identical yields for the latter two organisms. Also with respect to the specific substrate uptake rate and its kinetic properties, *V. parvula*, although using the methylmalonyl-CoA pathway, resembles *C. homopropionicum* more than *P. freudenreichii* (Table 3).

P. propionicus can also ferment ethanol to acetate plus propionate, according to the equation:

$$3\ CH_3CH_2OH + 2\ CO_2 \rightarrow CH_3COO^- + 2\ CH_3CH_2COO^- + 3\ H^+ + H_2O \quad (2)$$

$$\Delta G^{o\prime} = \quad 124\ \text{kJ per 3 mol ethanol}$$

and uses the methylmalonyl-CoA pathway [26]. *P. propionicus* exhibited different growth rates and growth yields when growing either with lactate or ethanol. The comparably low growth yield with lactate (about 2/3 of that of *P. freudenreichii*) may be due in part to its slow growth and the high proportion of energy going into the maintenance metabolism. Growth with ethanol, on the other hand, although substantially faster, produced far lower cell yields than growth with lactate, as expected from the lower total energy available in this transformation (Eq. 2). The fermentation pathways for both substrates do not differ by reaction steps associated with substrate-level phosphorylation [26]. Since there is obviously also no difference in fumarate reductase activity or in cytochrome content, the different cell yields cannot be attributed to different efficiencies of operation of this enzyme system either. It remains unclear how these differences are to be explained. Either the ferredoxin-dependent reductive carboxylation of acetyl-CoA to pyruvate requires metabolic energy, e.g. in a proton-driven reversed electron transport from NADH to ferredoxin, or the additional ATP yield during lactate degradation is due to energy conservation in lactate uptake, different from the assumed energy conservation in lactate excretion by lactic acid fermenting bacteria [27,28]. If this is so, this increases the metabolic flexibility of *P. propionicus*, which can also increase its ATP yield by enhanced acetate formation and interspecies hydrogen transfer to a methanogenic partner.

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