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Energy conservation in malolactic fermentation by *Lactobacillus plantarum* and *Lactobacillus sake*

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Abstract. A comparably poor growth medium containing 0.1% yeast extract as sole non-defined constituent was developed which allowed good reproducible growth of lactic acid bacteria. Of seven different strains of lactic acid bacteria tested, only *Lactobacillus plantarum* and *Lactobacillus sake* were found to catalyze stoichiometric conversion of L-malate to L-lactate and CO_2 concomitant with growth. The specific growth yield of malate fermentation to lactate at pH 5.0 was 2.0 g and 3.7 g per mol with *L. plantarum* and *L. sake*, respectively. Growth in batch cultures depended linearly on the malate concentration provided. Malate was decarboxylated nearly exclusively by the cytoplasmically localized malo-lactic enzyme. No other C_4-dicarboxylic acid-decarboxylating enzyme activity could be detected at significant activity in cell-free extracts. In pH-controlled continuous cultures, *L. plantarum* grew well with glucose as substrate, but not with malate. Addition of lactate to continuous cultures metabolizing glucose or malate decreased cell yields significantly. These results indicate that malo-lactic fermentation by these bacteria can be coupled with energy conservation, and that membrane energetization and ATP synthesis through this metabolic activity are due to malate uptake and/or lactate excretion rather than to an ion-translocating decarboxylase enzyme.

Key words: Malo-lactic fermentation – Bioenergetics – Transport-coupled energetization – Continuous culture – Malo-lactic enzyme – Oxalo-acetate decarboxylase

Fermentative conversion of L-malate to L-lactate is called malo-lactic fermentation (Kunkee 1967) and is a well-known and important process during the so-called "secondary" acid-degrading fermentation which follows the primary alcoholic fermentation in wine making (Davis et al. 1985; Wibowo et al. 1985). Since malic acid makes up about half of the total acid content of the must, malo-lactic fermentation contributes considerably to the deacidification of wine and, with this, to its flavour quality and stability. Already to the end of the last century, lactic acid bacteria were found to carry out this important reaction (Koch 1898, 1900; Müller-Thurgau and Osterwalder 1913).

Conversion of malate to lactate through malo-lactic fermentation does not yield products for further energy generation and, at least at first sight, the process does not provide an obvious advantage to the bacteria. Simultaneous fermentation of L-malate and D-glucose by a *Leuconostoc mesenteroides* strain in a complex medium did not result in a quantitative or qualitative change of the fermentation products or cell yields (Kandler et al. 1973), and it was concluded that malo-lactic fermentation does not provide energy for growth. This view is also maintained in textbooks (e.g. Gottschalk 1979), however, it is now expressed less definitively (Gottschalk 1986). It is assumed that the physiological significance of this fermentation which is wide-spread among lactic acid bacteria should be mainly the decrease of acidity to compensate excess proton activity (Doelle 1975).

Indications of energy conservation through malo-lactic fermentation was obtained recently by assays of ATP contents in lactic acid bacteria in the presence or absence of malate (Cox and Henick-Kling 1989). Since the free energy change of the decarboxylation reaction is small ($\Delta G^{0\prime} = -26.5$ kJ per mol; Thauer et al. 1977), energy conservation could be associated only with the generation of a chemiosmotic gradient across the cytoplasmic membrane. Fermentative energy conservation through decarboxylation of dicarboxylic acids has been reported in the recent past for oxalate (Dawson et al. 1980; Smith et al. 1985; Dehning and Schink 1989a), malonate (Dehning and Schink 1989b; Dehning et al. 1989), succinate (Schink and Pfennig 1982; Denger and Schink 1990), and glutarate (Matthies and Schink 1992). In all cases, a fraction of an ATP equivalent was conserved, and molar growth yields in the range of 0.9–3.5 g dry cell matter were observed.

In the present paper, various strains of lactic acid bacteria were checked for their ability to grow by malate decarboxylation to lactate.

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Materials and methods

Sources of organisms

Lactobacillus casei (DSM 20008), *Lactobacillus coryniformis* (DSM 20005), *Leuconostoc mesenteroides* (DSM 20343) and *Lactobacillus sake* (DSM 20017) were obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, FRG. *Lactobacillus plantarum* strain B 38 was kindly provided by Prof. F. Radler, Mainz, FRG, *Lactobacillus* spec. (DSM 20252) by Dr. N. Weiss, Weihenstephan, FRG, and *Streptococcus faecalis*, 10C1, by Dr. P. van Demark, Cornell University, Ithaca, New York, USA.

Media and growth conditions

For anaerobic cultivation of lactic acid bacteria, cystein-reduced (2.8 mM) mineral salts media with 0.1% yeast extract and further supplements were used. Media were prepared anoxically after Widdel and Pfennig (1981). The following components were used (per liter): a) medium with final pH 6.4: KH_2PO_4, 6.8 g; NH_4Cl, 1.0 g, $MgSO_4 \times 7\,H_2O$, 0.2 g; $MnSO_4 \times H_2O$, 0.05 g; b) medium with final pH 5.0: KH_2PO_4, 0.68 g; Na_2-succinate, 6.75 g; NH_4Cl, 1.0 g; $MgSO_4 \times 7\,H_2O$, 0.2 g; $MnSO_4 \times H_2O$, 0.05 g. Low-sodium media were prepared with the respective potassium salts. After autoclaving, media were supplemented with 1 ml of trace element solution SL 10 (Widdel et al. 1983) per liter, as well as 0.5 ml/l of ten-fold concentrated 7-vitamins solution (Widdel and Pfennig 1981). Substrates were added from concentrated sterile stock solutions before inoculation. Growth was followed via turbidity in 22-ml screw-cap tubes with a Spectronic 20 Spectrophotometer (Milton Roy, Rochester, NY, USA) at 450 nm or 546 nm. Culture purity was checked microscopically after growth in the above media or on agar plates with a complex medium (Evans and Niven 1951).

Experiments in pH-controlled batch and continuous culture were performed at 30 °C with the phosphate-buffered medium in an anaerobic chemostat system with 640-ml working volume without headspace. The pH was maintained at pH 5.7 with 1 MHCl or 1 M NaOH by a laboratory pH control set Type M 7822 NLC (Mostec, Liestal, Switzerland). Contamination of the reservoir was prevented by a heat trap; reservoir and trap were kept under N_2/CO_2 (90%/10%) at 50 mbar pressure. Growth was followed at 436 nm in 1-ml cuvettes.

Enzyme assay

Cell-free extracts were prepared from malate-grown cells harvested in the late log-phase, either by incubation with lysozyme ($2\ mg \cdot ml^{-1}$) in a 50 mM phosphate buffer, pH 7.2, with 1 mM $MgCl_2$ at 35 °C for 30 min, or by French press cell treatment at 6.9 MPa. After centrifugation at $20000 \times g$ for 20 min, the clear supernatant (crude extract) was used either directly for enzymatic studies, or was further subfractionated by centrifugation at $120000 \times g$ for 40 min.

Malo-lactic enzyme was measured via CO_2 formation either manometrically (Caspritz and Radler 1983) or by gas chromatography (Platen and Schink 1990). The test was carried out anoxically at 25 °C in 25-ml serum tubes sealed with butyl rubber stoppers; 300-µl samples were taken at intervals from the headspace after shaking. Standards were prepared with a 100 mM $NaHCO_3$ solution.

Lactate dehydrogenase (E.C. 1.1.1.27) and malate dehydrogenase (E.C. 1.1.1.37), were determined after Bergmeyer (1974). Oxaloacetate decarboxylase was assayed after Dimroth (1981), malate dehydrogenase, oxaloacetate decarboxylating ("malic enzyme", E.C. 1.1.1.39) after London and Meyer (1969).

Chemical analyses

Acetate and ethanol were assayed by gas chromatography as described (Dehning et al. 1989). DL-malate and DL-lactate were quantified either enzymatically (Bergmeyer 1984, 1985) or by capillary gas chromatography of their methyl esters with malonate as internal standard (Dehning and Schink 1989b).

Protein was quantified by a micro-biuret method (Zamenhoff 1957) with bovine serum albumine as standard.

Chemicals

All chemicals used were of analytical grade and obtained from Fluka, Neu-Ulm, and Merck, Darmstadt, FRG. Biochemicals were purchased from Boehringer, Mannheim, and Sigma, München, FRG. Gases were obtained from Messer-Griesheim, Frankfurt, FRG.

Results

Malate utilization by lactic acid bacteria

Lactic acid bacteria are usually cultivated in rather complex media (e.g. Evans and Niven 1951; de Man et al. 1960). Studies on a possible energy conservation from malate utilization required a comparably poor growth medium which allowed determination of small growth yield increases over background growth. Experiments with *Lactobacillus plantarum* revealed that a mineral medium supplemented with trace elements, 7 vitamins, and 0.1% yeast extract allowed reliable growth with constant growth yields with glucose ($Y = 24$–$28\ g \cdot mol\ glucose^{-1}$) up to 10 mM initial concentration. The medium could be buffered efficiently at pH 6.4 with 20 mM potassium phosphate, or at pH 5.0 with 20 mM sodium succinate.

Seven different strains of lactic acid bacteria were checked in succinate-buffered medium for utilization of malate. As shown in Table 1, only *Lactobacillus sake* and *L. plantarum* converted malate nearly stoichiometrically to lactate with significant growth. *L. casei* and *L. coryniformis* carried out basically the same fermentation but growth was considerably lower. No growth during lactate utilization was observed with *Lactobacillus* sp., *Leuconostoc mesenteroides*, or *Streptococcus faecalis*. Growth experiments at pH 6.4 with phosphate-buffered medium yielded basically similar results, but malate-dependent growth yields were slightly higher with *L. casei* and *L. coryniformis* (results not shown). Cultivation in low-sodium (<0.1 mM Na^+) media had no influence on growth rates or yields. *L. sake* and *L. plantarum* both used only the L-enantiomer of D,L-malate, and converted it exclusively to L-lactate. Contrary to *L. sake*, *L. plantarum* exhibited cell autolysis only after prolonged incubation for more than 2 days beyond growth cessation, and was therefore used in the following growth experiments.

Variation of yeast extract versus malate supply with *L. plantarum* (Fig. 1) demonstrated that yeast extract supported growth as energy and cell carbon source independent of malate addition. Malate, on the other

Table 1. Malate transformation and malate-dependent growth of various lactic acid bacteria at pH 5.0

	Malate provided [mM]	net OD_{450}[1]	Cell matter formed[2]	Malate consumed		Products formed [mM][3]	Y_{Malate} [g DM · mol^{-1}]
				L-Malate	D-Malate [mM]		
L. casei	30	0.076	22.8	15.0	0.2	15.9 DL-Lactate 0.4 Acetate	1.5
L. coryniformis	30	0.065	19.4	15.0	1.4	16.5 L-Lactate	1.2
L. plantarum	20	0.054	17.8	9.0	0.0	9.0 DL-Lactate 0.4 Acetate	2.0
L. sake	20	0.133	37.5	10.0	0.2	9.9 L-Lactate	3.7
Lactobacillus sp.	20	0.004	–	2.8	0.0	1.1 DL-Lactate	–
L. mesenteroides	30	0.014	–	6.1	0.0	3.4 DL-Lactate 0.4 Ethanol	–
S. faecalis	20	0.022	–	1.2	0.0	–	–

Experiments were carried out in 22-ml screw-cap tubes

[1] net OD is OD of the experimental tubes minus that reached in malate-free control tubes

[2] Cell dry matter was calculated via cell turbidity using the experimentally determined conversion factors 0.1 OD_{450} = 29.7 mg dry matter per liter *(L. mesenteroides)*, 33.5 mg dry matter per liter (*L.* spec.), 36.0 mg dry mass per liter *(S. faecalis)*, 30.0 mg dry mass per liter *(L. casei)*, 29.8 mg dry mass per liter *(L. coryniformis)*, 33.1 mg dry mass per liter *(L. plantarum)*, 28.2 mg dry mass per liter *(L. sake)*

[3] D,L-Lactate, acetate, ethanol and propionate were assayed in all samples. Net product concentrations were calculated as differences of test tubes vs. malate-free control tubes. Concentrations <0.3 mM are not listed

hand, could serve as energy source only if at least 0.1% yeast extract was provided which was probably mainly assimilated into cell material. Similar results were obtained with *L. sake* (not shown).

Growth of *L. plantarum* with 15 mM L-malate in the presence of 0.1% yeast extract was followed in pH-controlled batch fermentor cultures at pH 5.7. Initial growth after inoculation was fast and mainly due to yeast extract fermentation (Fig. 2a). In a second growth phase between 5 and 15 h after inoculation, malate was converted stoichiometrically to lactate and trace amounts of acetate. Growth was considerably slower through this phase, and ceased nearly completely after about 7 mM malate was degraded. In the last phase, the leftover malate was converted to lactate without significant growth (Fig. 2a). Also in this culture system, malate-dependent growth correlated strictly with the amount of malate provided, and lactate was found as nearly only product, together with traces of acetate (Fig. 2b).

Continuous culture and product inhibition experiments

In continuous culture with 5 mM glucose as substrate (results not shown), *L. plantarum* grew well with nearly constant growth yields at various dilution rates. The maximum growth rate reached reproducibly was 0.483 h^{-1} (μ_{max} = 1.4 h). The growth yield was between 24.8 and 26 g dry matter per mol glucose; the maintenance coefficient m_E was determined to $1.67 \cdot 10^{-4} \pm 0.3 \cdot 10^{-4}$ mol glucose per gram dry matter.

Similar experiments with D,L-malate as substrate yielded basically different results. Fig. 3 shows the dependence of optical density, leftover substrate, and products formed on increasing concentrations of malate provided in the reservoir. In all cases, equilibrium states are plotted which were reached after at least 3 full medium exchanges at a dilution rate of 0.027 h^{-1} corresponding to about 0.5 μ_{max} (μ_{max} = 0.066 h^{-1} ± 0.005 h^{-1} was determined for growth with malate in washout experiments). At

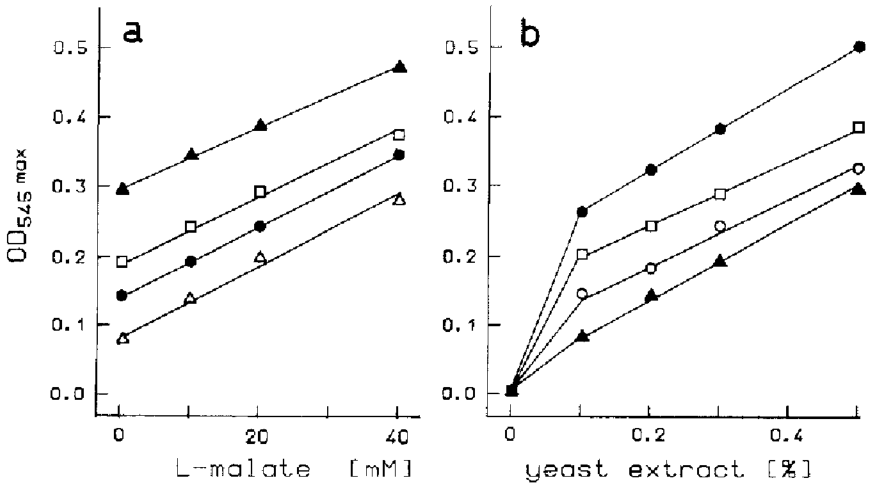

Fig. 1a, b. Growth of *Lactobacillus plantarum* in minimal medium, pH 5.0, with L-malate and yeast extract. Maximum OD values reached are shown. **a** Variation of L-malate provided with constant amounts of yeast extract. (△) 0.1%, (●) 0.2%, (□) 0.3%, (▲) 0.5% yeast extract in the medium background. **b** Variation of yeast extract provided with constant amounts of malate. (▲) 0 mM, (○) 10 mM, (□) 20 mM, (4) 40 mM L-malate in the medium background

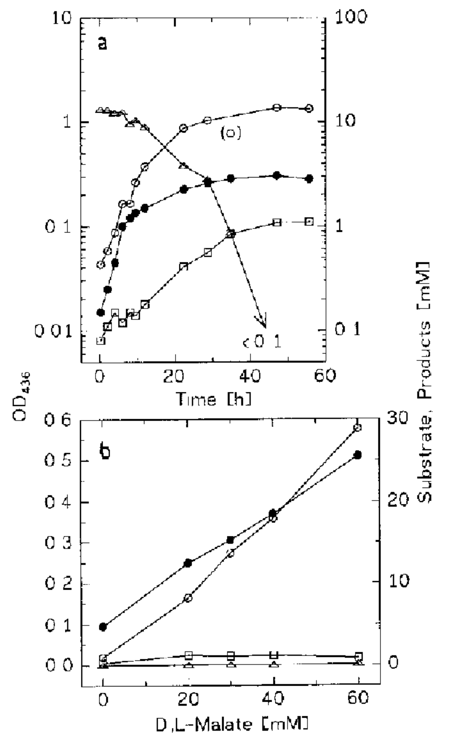

Fig. 2a. Growth of *Lactobacillus plantarum* in pH-controlled batch culture at pH 5.7 with 30 mM D,L-malate in the presence of 0.1% yeast extract. Optical density, product and substrate concentration are plotted on a logarithmic scale. (●) Optical density, (△) L-malate, (○) L-lactate, (□) acetate. **b** Malate-dependent growth of *Lactobacillus plantarum* in pH-controlled batch culture. (●) Optical density, (△) L-malate, (○) L-lactate, (□) acetate

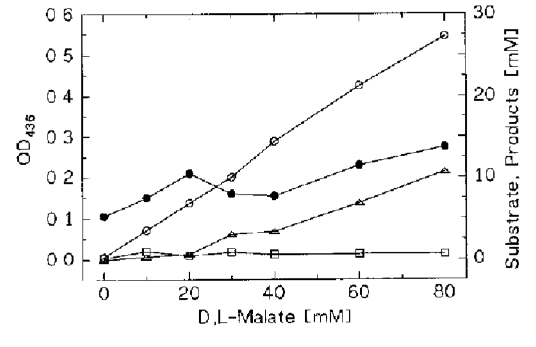

Fig. 3. Growth of *L. plantarum* with D,L-malate at various concentrations in continuous culture at pH = 5.7. The dilution rate D was 0.027 h^{-1}. (●) Optical density, (△) L-malate, (○) L-lactate, (□) acetate

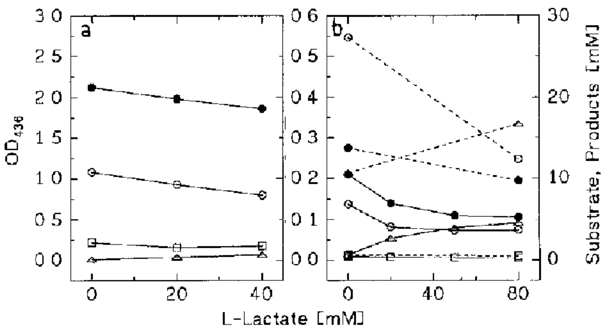

Fig. 4a, b. Effect of added L-lactate on growth and substrate utilization by *Lactobacillus plantarum* in continuous culture at pH = 5.7; **a** 5 mM D-glucose as substrate, dilution rate D = 0.184 h^{-1}; **b** 10 mM *(solid lines)* or 40 mM *(dashed lines)* D,L-malate as substrate, dilution rate D = 0.027 h^{-1}. (●) Optical density, (△) L-malate, (○) L-lactate, (□) acetate

D,L-malate concentrations lower than 20 mM, the optical density increased slightly less than in batch cultures, with a specific growth yield of 1.44 g dry matter per mol malate. L-malate was used up nearly completely, and the L-lactate concentration increased linearly (Fig. 3). At higher malate concentrations provided, the formed L-lactate increased as well, but more and more L-malate accumulated unused in the fermentor. The optical density did not increase any further but remained in the same range as with 20 mM D,L-malate.

To check for effects of accumulating lactate on substrate conversion and cell yields, *L. plantarum* was cultivated in pH-controlled continuous cultures at pH 5.7 with glucose or malate as substrate, in the presence of varying amounts of L-lactate in the reservoir. With 5 mM glucose as substrate, 20 mM added L-lactate decreased growth by 6% and lactate production by about 12% (Fig. 4a). Glucose was used up completely to the detection limit. The effect of added lactate on malate utilization was more dramatic (Fig. 4b): With 10 mM L-malate as substrate, 20 mM added L-lactate decreased growth yields by 33% and lactate formation by 41%; the remnant malate accumulated in the reactor. Higher lactate additions decreased yields and product formation further, but never completely. With 40 mM L-malate as substrate, similar effects were observed (Fig. 4b). Acetate was not formed to significant amounts.

Enzyme measurements

Enzymes possibly involved in malate conversion to lactate were determined in crude cell extracts of *L.*

Table 2. Specific activities of malate-transforming enzymes in cell-free extracts of *Lactobacillus sake* and *Lactobacillus plantarum*. All enzyme activities were found to more than 96% in the cytoplasmic fraction

Enzyme	E.C. number	*L. sake*	*L. plantarum*
Malate dehydrogenase	1.1.1.37	<0.1	0.035
Malate dehydrogenase, oxalo-acetate-decarboxylating	1.1.1.40	<0.1	0.16
Oxaloacetate decarboxylase	4.1.1.3	n.d.	<0.1
Malo-lactic enzyme	?	22.0	0.90

Enzyme activities are listed as µmol · min^{-1} · mg^{-1} protein

through the entire growth curve: After transformation of about 60–70% of the provided malate, growth ceased while malate was still transformed to lactate. Obviously, decarboxylation-dependent energy conservation operated best at a high substrate-to-product ratio, and became inefficient when this ratio fell under a certain limit. This observation gave a first hint that substrate import or product export could have key functions in this type of energy conservation.

We therefore started growth experiments in a chemostat because in this culture system the bacterium has to operate always at limiting substrate concentration against the full corresponding product concentration (Veldkamp 1977). Growth of *L. plantarum* in the chemostat was not directly correlated with the malate concentration provided in the reservoir, but exceeded yeast extract-dependent growth only slightly. Nonetheless, malate was still decarboxylated to lactate, however, not completely, and leftover malate accumulated in the reactor at a certain ratio over the lactate formed, as typical of cultures under the influence of product inhibition (Pirt 1975). Since this effect was expressed much more severely with malate than with glucose as substrate, it is obvious that product efflux plays an essential and specific role in energy conservation by malolactic fermentation. Energy conservation coupled to lactate efflux in lactic acid bacteria has been hypothesized repeatedly on the basis of convincing indirect experimental evidence (Michels et al. 1979; Otto et al. 1980; ten Brink and Konings 1982; Konings and Veldkamp 1980, 1983). On the other hand, *Propionigenium modestum* which conserves decarboxylation energy by a primary sodium pump can be grown in continuous culture at constant yields at various substrate concentrations (B. Schink, unpublished).

Substrate import, on the other hand, cannot contribute significantly to the energy budget in malo-lactic fermentation because the substrate affinities of the described malolactic enzymes are far too low (K_m for malate at 9.5 and 16.7 mM with *L. plantarum* and *L. mesenteroides*, respectively; Caspritz and Radler 1983; Lonvand-Funel and Strasser de Saad 1982) to establish an energy-yielding substrate gradient across the membrane. Rather, malate transport across the cytoplasmic membrane appears to be an electroneutral symport with protons, at least in the yeast *Hansenula anomala* (Corte-Real and Leão 1990). One could also think of an electrogenic malate/lactate antiporter as a means of energy conservation as suggested by Poolman (1990), analogous to the oxalate/formate antiporter of *Oxalobacter formigenes* (Anantharam et al. 1989). For the reason mentioned before, such a system would need to recruit its energy mainly from a high inside-to-outside lactate gradient in the range of 1000 : 1 in order to obtain the minimum energy unit of one third of an ATP equivalent (around 20 kJ $\cdot$ mol^{-1}) for ATPase-dependent ATP synthesis (Schink 1990). Obviously, this is possible only at an early stage in batch culture and hardly ever in continuous culture. The growth yields of malo-lactic fermentation determined in batch culture are mainly established during the initial substrate conversion phase, therefore, and are in general smaller than those observed with decarboxylation fermentations depending on primary sodium pumps.

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