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Electrogenic L-Malate Transport by *Lactobacillus plantarum*: a Basis for Energy Derivation from Malolactic Fermentation

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L-Malate transport in *Lactobacillus plantarum* was inducible, and the pH optimum was 4.5. Malate uptake could be driven by an artificial proton gradient (ΔpH) or an electroneutral lactate efflux. Because L-lactate efflux was unable to drive L-malate transport in the absence of a ΔpH, it did not appear that the carrier was a malate-lactate exchanger. The kinetics of malate transport were, however, biphasic, suggesting that the external malate concentration was also serving as a driving force for low-affinity malate uptake. Because the electrical potential (ΔΨ, inside negative) inhibited malate transport, it appeared that the malate transport-lactate efflux couple was electrogenic (net negative) at high concentrations of malate. De-energized cells that were provided with malate only generated a large proton motive force (>100 mV) when the malate concentration was greater than 5 mM, and malate only caused an increase in cell yield (glucose-limited chemostats) when malate accumulated in the culture vessel. The use of the malate gradient to drive malate transport (facilitated diffusion) explains how *L. plantarum* derives energy from malolactic fermentation, a process which does not involve substrate-level phosphorylation.

Wine contains from 20 to 60 mM L-malate, but much of the malate can be degraded by lactic acid bacteria (1, 9, 22). The stoichiometric conversion of L-malate to L-lactate and carbon dioxide deacidifies wine (9, 10). If malate is not utilized before bottling, malate decarboxylation can cause hazes, tartrate precipitation, and carbonation (9). In cucumber fermentations, carbon dioxide arising from the malolactic fermentation can cause bloating and undesirable changes in texture (29).

Escherichia coli, *Enterococcus faecalis*, and *Lactobacillus casei* can convert L-malate to pyruvate via the malic enzyme, but similar activity could not be detected in *Lactobacillus plantarum* (15, 41). Subsequent work showed that L-malate was converted directly to L-lactate by a single enzyme, the malolactic enzyme (4). The malolactic enzyme is produced by a variety of bacteria including lactobacilli, leuconostocs, pediococci, and streptococci (4, 38). *Leuconostoc oenos* is often the predominant malate fermenter in wine, but this species grows slowly and is usually present at low numbers in the early stages of vinification (11, 27). *L. plantarum* grows more rapidly, and this highly acid-tolerant bacterium can utilize malate at a rapid rate (20).

There have been suggestions that malolactic fermentation, a process that does not involve substrate-level phosphorylation, may provide metabolic energy (33–35, 37). Cox and Henick-Kling (7, 8) observed an increase in ATP during malolactic fermentation, and it appeared that a proton motive force (Δp) was involved. The mechanism of energy transduction by malolactic fermentation has not been elucidated. Results presented here show that electroneutral lactate efflux from *L. plantarum* created a chemical gradient of protons (ZΔpH). Malate transport could be driven by a ZΔpH, but it appeared that the stoichiometry of malate-proton symport was variable. At low malate concentrations, malate transport and lactate efflux constituted an electroneutral process that was unable to generate either an electrical potential (ΔΨ) or a ZΔpH. When the malate concentration was increased, the ratio of malate^{-1} to proton transported appeared to increase and *L. plantarum* was able to create both a ΔΨ and a ZΔpH.

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MATERIALS AND METHODS

Cell growth. *L. plantarum* L11a1 was obtained from the Australian Wine Institute, Adelaide, Australia. Cultures were grown at 30°C in a modified MRS medium (7) containing 4 g of D,L-malate per liter. The pH was adjusted to 6.5 with KOH, and glucose (30 mM final concentration) was added after autoclaving. Continuous cultures (180-ml vessel, 0.1 h^{-1}, 30°C, under CO_2) were grown with 8.0 mM glucose, and the pH was maintained at 4.5 by the addition of 1 N KOH. After 5 days, 5 mM L-malate was added to the medium reservoir. At 2-day intervals (99% turnover of culture medium), the concentration of L-malate in the medium reservoir was gradually increased to 75 mM. Cells were harvested by centrifugation (10,000 × *g*, 5 min, 5°C) and washed twice in 50 mM potassium phosphate (pH 6.5). Concentrated cell suspensions and cell supernatants were stored at −15°C.

Malolactic fermentation by washed cells. Exponential cultures were washed in 50 mM K_2HPO_4 (pH 6.5) and resuspended in MES-phosphate (100 mM morpholineethanesulfonic acid [MES], 50 mM K_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 40 μM $MnSO_4 \cdot H_2O$). The washed cells (0.3 mg of protein per ml) were treated with glucose (22 mM final concentration) and various inhibitors (iodoacetate, dicyclohexylcarbodiimide, or tetrachlorosalianilide [TCS]) where specified. Malate fermentation was initiated by the addition of L-malic acid (20 mM final concentration). The pH was adjusted from 6 to 3 by adding concentrated HCl to the MES-phosphate buffer. The

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washed cell suspensions were incubated at 30°C for 0 to 2 h. The fermentation was terminated by centrifugation (1.5 ml, 13,000 × *g*, 1 min, 23°C), and the supernatant was analyzed for L-malic acid.

Transport assays. Cells were harvested (12,000 × *g*, 5 min, 4°C) during exponential growth (0.15 mg of protein per ml) and washed twice in MES-phosphate buffer with the pH adjusted to 4.5 with HCl. De-energized cells were prepared by 30 min of incubation with iodoacetate (500 μM). The cells were resuspended in the same buffer and concentrated (12 to 24 mg of protein per ml). Concentrated cells (4 μl) were diluted 50-fold into 200 μl of buffer (30°C), and transport was initiated by the addition of 100 to 400 nCi of L-[U-^{14}C]malic acid (53 mCi/mmol) or 100 nCi of L-[U-^{14}C]lactic acid (169 mCi/mmol). After 0 to 60 s, transport was terminated by the addition of ice-cold LiCl (2 ml, 100 mM) and rapid filtration (0.45-μm-pore-size cellulose-nitrate filter). The filter was washed once with 2.0 ml of LiCl and dried for 20 min at 105°C, and the radioactivity was determined by liquid scintillation counting.

Artificial membrane potentials. Cells were de-energized for 30 min with 500 μM iodoacetate. Acetate-loaded cells (80 mM potassium acetate, 100 mM MES, 23.3 mM K_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 500 μM iodoacetate [pH 4.5]) were diluted into buffer lacking acetate (100 mM MES, 50 mM K_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 500 μM iodoacetate [pH 4.5]) to create an artificial $Z\Delta pH$. An artificial $\Delta\Psi$ was generated by loading valinomycin (5 μM)-treated cells with potassium (100 mM MES, 50 mM K_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 500 μM iodoacetate [pH 4.5]) and diluting them into sodium buffer (100 mM MES, 50 mM Na_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 500 μM iodoacetate [pH 4.5]). A L-lactate diffusion potential was created by loading cells with L-lactate (100 mM MES, 50 mM K_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$, 500 μM iodoacetate, 50 mM L-lactic acid [pH 4.5]) for 30 min on ice and diluting into the same buffer without lactate. Transport was initiated by a 50-fold dilution of concentrated cells into buffer containing the radioactive malate or lactate (see above).

Δp. Intracellular pH was determined by an acid distribution method with the Henderson-Hasselbalch equation. Growing cells (2.0 ml, 0.2 to 0.6 mg of protein, pH 6.5 to 3.5) or iodoacetate-treated cells that were provided with L-malate (2.0 ml, 0.6 mg of protein, pH 4.5, 1 mM iodoacetate, 20 mM L-malate, pH adjusted to 4.5 with Tris) were incubated with either [carboxyl-^{14}C]salicylate (500 nCi, 56.5 mCi/mmol), [7-^{14}C] benzoate (500 nCi, 21.8 mCi/mmol), [U-^{14}C]taurine (500 nCi, 35 mCi/mmol), or 500 nCi of ^3H_2O (5 min, 30°C). The cell suspensions were centrifuged (0.9-ml samples, 13,000 × *g*, 5 min) through 0.35 g of silicon oil (70:30 mixture of Dow Corning 550 and Dow Corning 556; William F. Nye, Inc., New Bedford, Mass.), and the supernatant (20 μl) was removed for scintillation counting. The tubes were then frozen (−15°C), and the pellets were removed with dog nail clippers. The cell pellets were dissolved in scintillation fluid and counted. Intracellular volume (3.2 μl/mg of protein) was estimated from the difference between [^{14}C]taurine and ^3H_2O, and corrections were made for extracellular benzoate or salicylate. The $Z\Delta pH$ was calculated as 60 mV × ΔpH. The $\Delta\Psi$ was estimated from the distribution of tetra-[U-^{14}C] phenylphosphonium bromide ([^{14}C]TPP^+; 250 nCi, 30 mCi/mmol) by using the Nernst equation. Nonspecific TPP^+ binding was estimated from cells that were treated with nigericin and valinomycin (5 μM each).

Over short time intervals (0 to 60 s), the $\Delta\Psi$ was determined by a membrane filtration procedure. Cell suspensions (200 μl) that had been incubated with [^{14}C]TPP^+ (100 nCi, 30 mCi/mmol) were diluted with ice-cold buffer (2 ml, 50 mM MES, 50 mM morpholinepropanesulfonic acid, 10 mM $MgCl_2 \cdot 6H_2O$, pH adjusted to 7.0 with choline chloride) and collected on cellulose-acetate filters (0.45-μm pore size). The filters were then washed with another 2 ml of buffer, and the radioactivity was counted as described above.

Membrane vesicles. Right-side-out membrane vesicles were prepared by mutanolysin-lysozyme digestion and osmotic lysis as previously described (23, 39).

Analyses. L-Lactate, D-lactate, L-malate, and glucose were determined enzymatically (3, 14, 16, 31). Cell protein was measured by the Lowry et al. method after hydrolysis (0.2 N NaOH, 15 min, 100°C) (28).

Materials. Radiolabeled benzoate and ^3H_2O were supplied by NEN, Wilmington, Del. Radiolabeled salicylate was obtained from ICN, Inc., Costa Mesa, Calif. All other radiolabeled chemicals were supplied by Amersham, Arlington Heights, Ill. TCS was obtained from Kodak, Rochester, N.Y.

RESULTS

Glucose and malolactic fermentation. Batch cultures of *L. plantarum* L11a1 grew rapidly in modified MRS medium, and the glucose was converted to D,L-lactic acid. The doubling time (70 min, pH 6.5) increased as the extracellular pH declined, but growth was observed at pH values as low as 3.5. As the extracellular pH declined, L11a1 maintained a relatively constant ΔpH (approximately 1.0 unit) across the cell membrane, and the intracellular pH declined. When the extracellular pH decreased, there was a reduction in the $\Delta\Psi$, and little TPP^+ was accumulated at pH 3.5.

Batch cultures of *L. plantarum* fermented L-malate, but L-malate alone could not support growth. Inocula that had been cultivated in media containing D,L-malate fermented L-malate at a rapid rate, but cells that were grown in the absence of L-malate did not have the malolactic enzyme and were unable to utilize L-malate. Induced cells (grown with D,L-malate) that were washed and energized with glucose utilized L-malate at a rate of 760 nmol/min/mg of protein, but the utilization rate eventually declined (Fig. 1). Iodoacetate, an inhibitor of glycolysis, and the ATPase inhibitor dicyclohexylcarbodiimide had no effect on malolactic fermentation. However, cells treated with TCS, a protonophore, fermented L-malate at a slower rate (350 nmol/min/mg of protein).

Malate transport by resting and glucose-energized cells. When exponentially growing cells were washed with buffer (100 mM MES, 50 mM K_3PO_4, 10 mM $MgSO_4 \cdot 7H_2O$ [pH 6.5]) and incubated with 10 μM L-[^{14}C]malate at pH 3.0 to 6.5, the transport rate was maximal at pH 4.5, the pH at which monovalent malate predominates (Fig. 2a). The maximum rate of L-malate transport corresponded with the maximum rate of malate fermentation (Fig. 2b). Glucose-energized cells took up L-malate at a faster rate than did nonenergized cells, but in either case uptake was inhibited by iodoacetate or TCS (Fig. 3).

Glucose-energized cells that had been grown in the absence of malate did not take up L-malate, and L-malate was a stronger inducer than D-malate in these cells (Table 1). Some induction was also noted with citrate and L-tartrate, but D-tartrate, succinate, fumarate, and aspartate were poor inducers. Cells grown at pH 3.5 and energized with glucose had fourfold more L-malate transport activity than did cells grown at pH 6.5. Competition studies (5 mM unlabeled acid

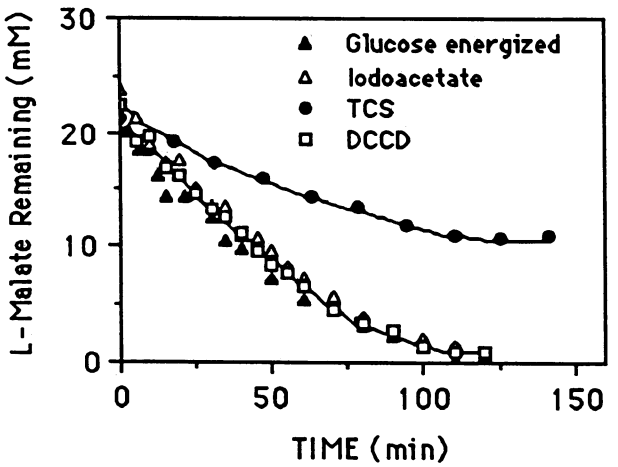

FIG. 1. Malolactic fermentation by washed cells (0.3 mg of protein per ml, pH 4.5), which were energized with glucose (22 mM, 5 min), de-energized with iodoacetate (1 mM, 30 min), or treated with TCS (5 μM, 10 min) or dicyclohexylcarbodiimide (DCCD) (100 μM, 30 min).

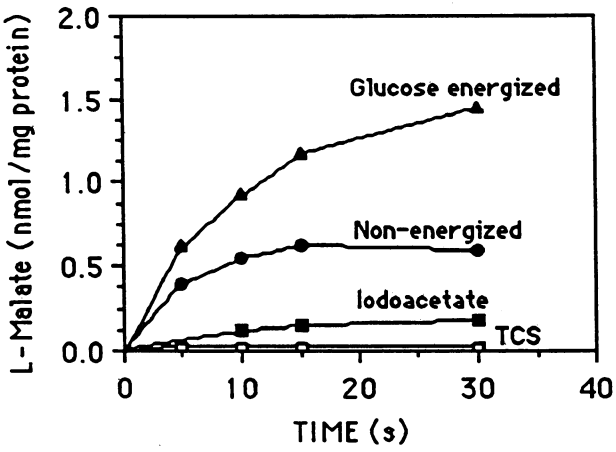

FIG. 3. L-Malate (10 μM, pH 4.5) transport by washed cells, which were energized with glucose (22 mM, 5 min), nonenergized, de-energized with iodoacetate (500 μM, 30 min), or treated with TCS (5 μM, 10 min).

versus 100 μM L-[^{14}C]malate) indicated that the L-malate carrier was stereospecific and not significantly inhibited by D-malate (Table 2). A 50-fold excess of itaconate and *meso*-tartrate inhibited L-malate transport by less than 60%, and maleate, succinate, citrate, L-tartrate, D-tartrate, malonate, and bromosuccinate had almost no effect. *L. plantarum* L11a1 was not able to take up [^{14}C]succinate, even if the growth medium contained succinate.

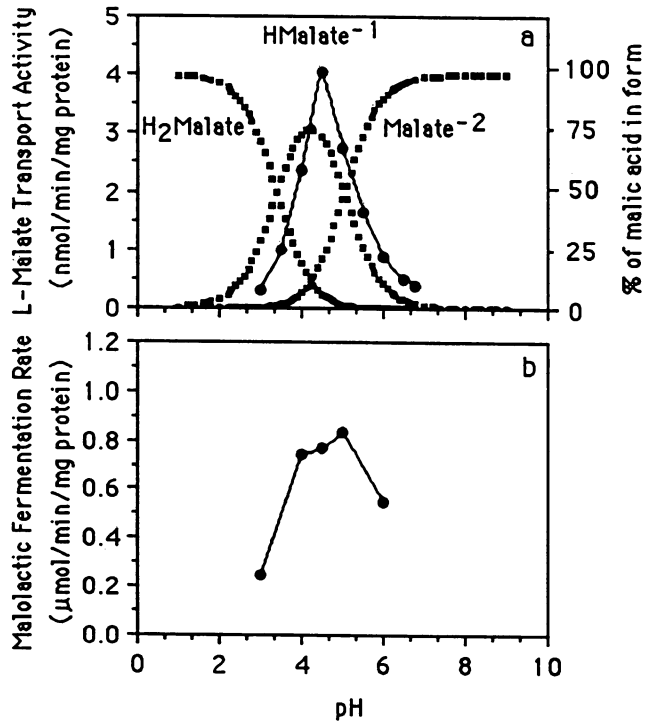

FIG. 2. Effect of extracellular pH on L-malate transport and fermentation by resting cells. (a) L-Malate transport rate was estimated after 10 s of uptake of 10 μM L-[^{14}C]malate, and the dissociation curves of malate (■) were calculated from the Henderson-Hasselbalch equation. (b) Effect of pH on malolactic fermentation (20 mM L-malate) by washed cells.

Malate-proton symport. Washed cells that were de-energized with iodoacetate, loaded with acetate, and diluted 50-fold into acetate-free buffer transported L-malate faster than did glucose-energized cells (Fig. 3), and no transport was observed in the absence of a $Z\Delta pH$ (Fig. 4a). L-Lactate-loaded cells also took up L-malate, but only if there was a concentration gradient of lactate across the cell membrane (Fig. 4b). Cells that were treated with TCS were unable to use a L-lactate diffusion potential to drive L-malate transport. When cells were loaded with L-lactate and acetate and diluted into buffer containing only acetate, there was little uptake of L-malate, and it appeared that acetate influx was counteracting the lactate diffusion potential. *L. plantarum* vesicles accumulated TPP^+ in response to an artificial $\Delta\Psi$, but an artificial $\Delta\Psi$, $Z\Delta pH$, and lactate gradient could not drive L-malate transport.

L-Lactate translocation. De-energized cells could not take up L-[^{14}C]lactate, but transport was observed when an artificial $Z\Delta pH$ was imposed (Fig. 5a). An artificial $\Delta\Psi$ could not drive L-lactate uptake. When cells were loaded with 50

TABLE 1. Induction of L-malate transport by various carboxylic acids and aspartate[a]

Analog[b]	Induction of transport (%)[c]
L-Malate	100
D,L-Malate	82
D-Malate	66
Citrate	42
L-Tartrate	23
Succinate	17
Fumarate	12
D-Tartrate	11
Aspartate	7
None	0

[a] Rates were estimated after 10 s of uptake of L-[^{14}C]malate (10 μM, pH 4.5) by glucose-energized cells.

[b] Cells were grown in modified MRS medium with 4 g of the potential inducer per liter and 30 mM glucose.

[c] Based on a rate of 6.25 nmol/min/mg of protein.

TABLE 2. Inhibition of L-malate transport by various malate analogs

Analog[a]	Inhibition (%)[b]
D-Malate	30
Itaconate	52
meso-Tartrate	31
Maleate	14
Succinate	5
Citrate	None
L-Tartrate	None
D-Tartrate	None
Malonate	None
Bromosuccinate	None

[a] Rates were estimated after 10 s of uptake of 100 μM L-[^{14}C]malate (200 nCi) and 5 mM of analog at pH 4.5. Cells were energized with glucose (22 mM, 5 min).

[b] Based on a control value of 46 nmol/min/mg of protein.

mM cold L-lactate and diluted into L-[^{14}C]lactate, there was a flux of [^{14}C]lactate that resembled counterflow (Fig. 5b). L-[^{14}C]lactate accumulation was reversed by the addition of 10 mM unlabeled L-lactate, but flux could not be demonstrated in cells that were treated with TCS. Valinomycin-treated, potassium-loaded cells that were diluted 50-fold into potassium-free buffer generated a $\Delta\Psi$ of 100 mV (filter method), in accordance with the Nernst equation, but L-lactate-loaded cells that were diluted 50-fold into buffer lacking lactate did not take up TPP^+ (pH 4.5 or 6.5).

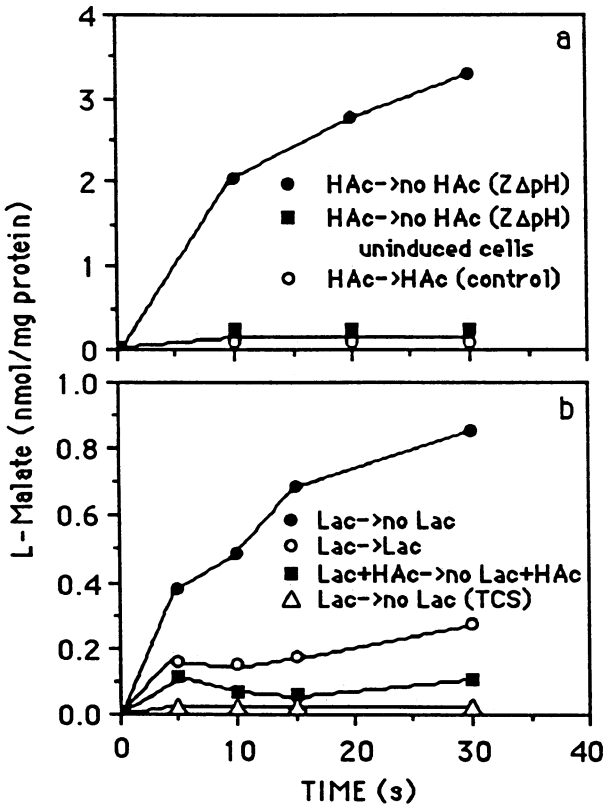

FIG. 4. (a) Effect of an artificial $Z\Delta$pH on malate transport (L-malate; 10 μM, pH 4.5) by de-energized cells that had been grown in the presence or absence (uninduced) of malate. (b) Effect of a lactate gradient (Lac) and either acetate (HAc) or TCS on L-malate transport.

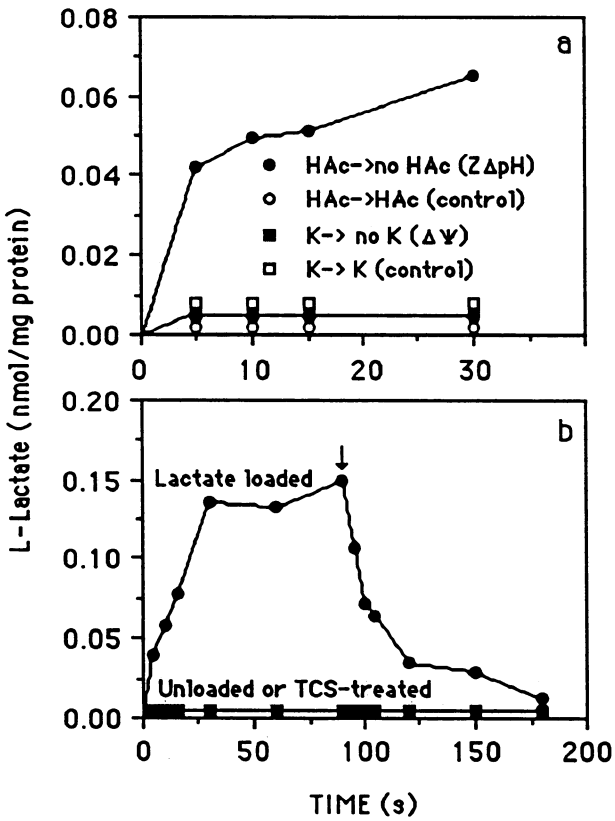

FIG. 5. (a) Effect of acetate (HAc) diffusion ($Z\Delta$pH) or potassium diffusion from valinomycin-treated cells ($\Delta\Psi$) on L-lactate (2.9 μM, pH 4.5) uptake. (b) Flux of L-lactate into de-energized cells loaded with 50 mM unlabeled L-lactate and diluted 50-fold in 2.9 μM L-[^{14}C]lactate. At 90 s (arrow), 10 mM unlabeled external L-lactate was added. The effect of 5 μM TCS on L-lactate flux is also shown.

Electrogenic malate transport. When de-energized cells were loaded with potassium and diluted into sodium phosphate buffer, the artificial $\Delta\Psi$ could not drive L-malate uptake (Fig. 6a). This result indicated that malate^{-1} was not taken up in symport with more than one proton, but the transport rate of these valinomycin-treated cells was low. Subsequent experiments showed that valinomycin treatment (potassium flux) inhibited $Z\Delta$pH-driven malate transport even when an artificial $\Delta\Psi$ was not created (Fig. 6b).

At pH 6.0 *L. plantarum* batch cultures had a $\Delta\Psi$ of approximately 80 mV, and at this pH the L-malate transport rate was low (Fig. 2a). When glucose-energized cells were treated with TPP^+, a lipophilic cation which would dissipate $\Delta\Psi$, the rate of L-malate uptake increased (Fig. 7). This latter result suggested that the $\Delta\Psi$ (inside negative) was inhibitory. At pH 4.5 the $\Delta\Psi$ was low, and TPP^+ was no longer stimulatory (data not shown).

Low-affinity L-malate transport. When glucose-energized cells were incubated with increasing concentrations of L-malate, the Eadie-Hofstee plot was biphasic and it appeared that the malate gradient was also driving transport (Fig. 8). Since the slope of the Hill plot (log $v/V_{max} - v$ versus log malate) was approximately 1, the biphasic kinetics could not be explained by additional binding sites (data not shown). The high-affinity system had a K_t of 540 μM and

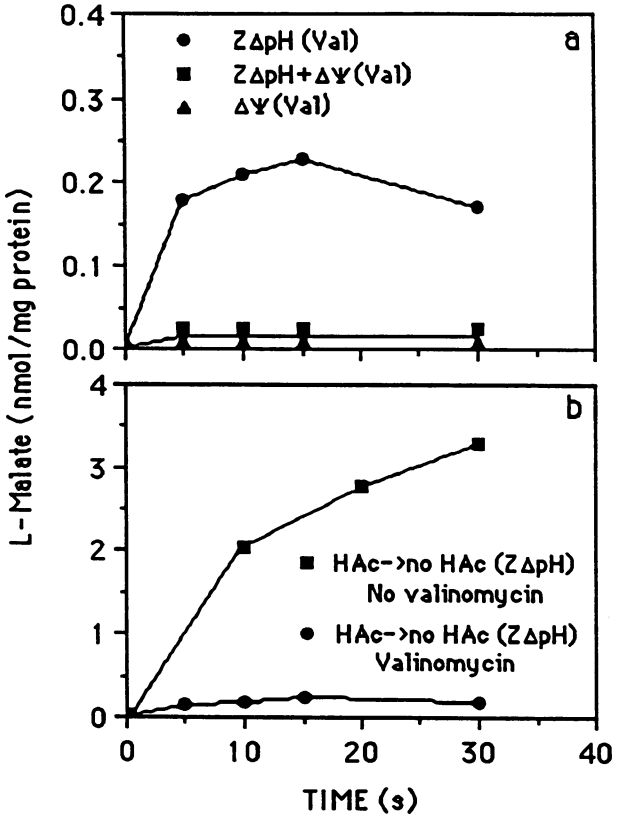

FIG. 6. (a) Effect of acetate (HAc) diffusion ($Z\Delta pH$) or potassium diffusion ($\Delta\Psi$) on malate transport by valinomycin (Val)-treated cells. (b) Effect of valinomycin treatment on $Z\Delta pH$-driven malate transport. Malate was present at 10 μM, and the pH was 4.5.

a V_{max} of 250 nmol/min/mg of protein. The K_t of low-affinity transport was 30-fold greater, and the V_{max} was 750 nmol/min/mg of protein. Only the low-affinity system had a high enough V_{max} to account for the malate fermentation of batch cultures (Fig. 2b).

L-Malate-dependent Δp generation. De-energized cells (pH 4.5) had a $Z\Delta pH$ of only 35 mV, and there was essentially no

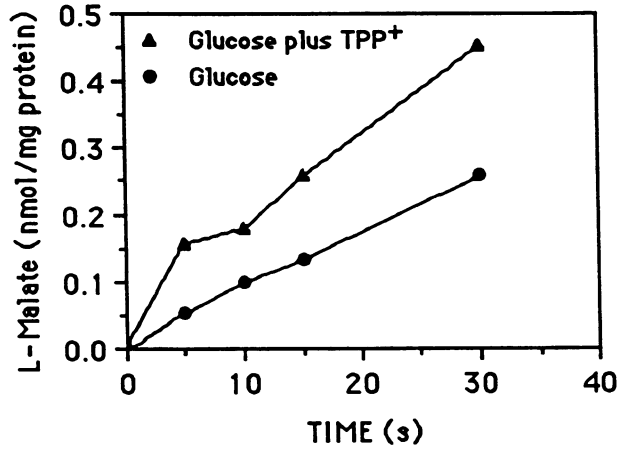

FIG. 7. Effect of TPP^+ (500 μM) on L-malate transport by glucose-energized cells at pH 6.0 (18 μM L-malate).

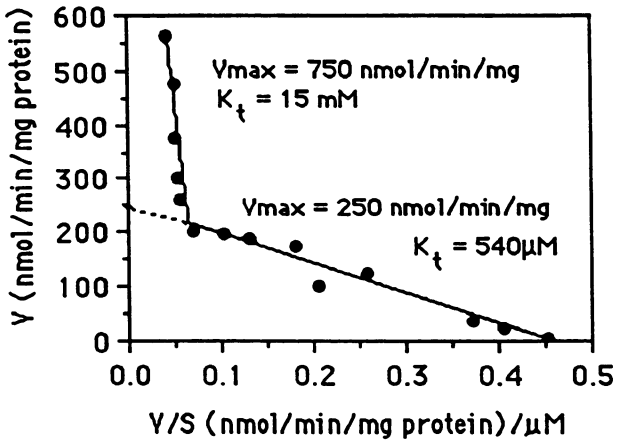

FIG. 8. Eadie-Hofstee plot of L-malate transport by glucose-energized cells (22 mM, 5 min). The initial rates were estimated after 10 s of uptakes of L-[^{14}C]malate (10 μM to 13.8 mM, pH 4.5).

$\Delta\Psi$. When 20 mM L-malate was added, the $\Delta\Psi$ increased to 110 mV (Fig. 9a). The $\Delta\Psi$ eventually decreased, giving rise to a proportional increase in $Z\Delta pH$. The total Δp remained relatively constant (approximately 160 mV) until malate was depleted (data not shown). When malate was decreased from 40 to 5 mM, there was little change in the $\Delta\Psi$, but lower

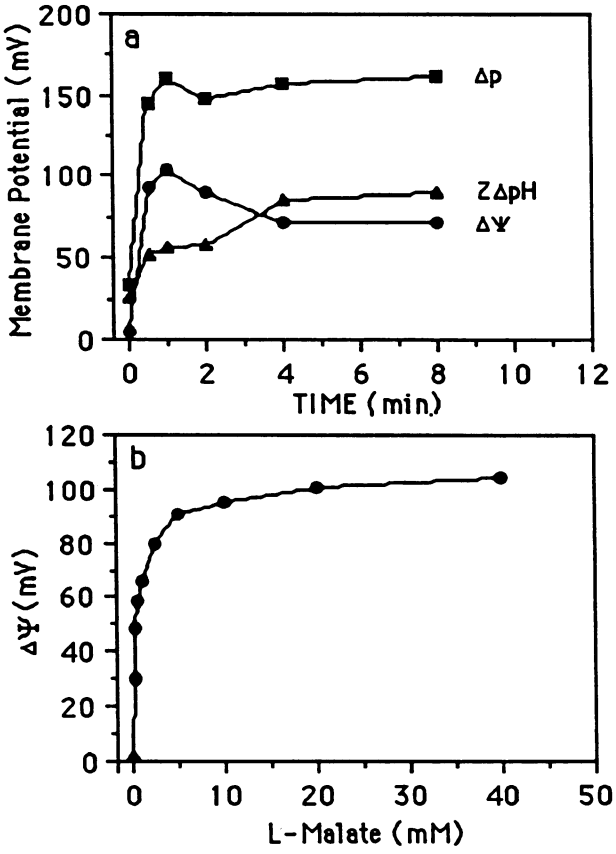

FIG. 9. (a) Generation of $Z\Delta pH$, $\Delta\Psi$, and Δp by de-energized cells that were provided with 20 mM L-malate at pH 4.5. (b) Effect of the extracellular L-malate concentration on the $\Delta\Psi$ (30 s).

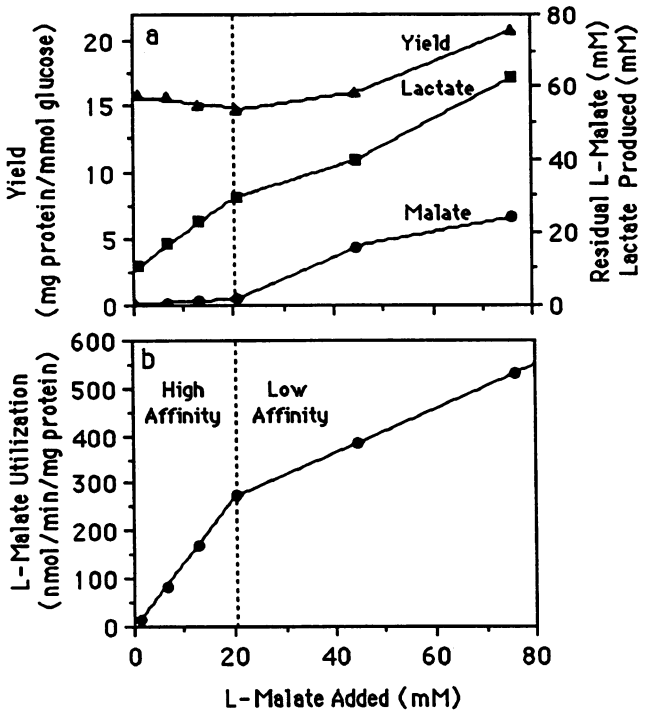

FIG. 10. (a) Effect of L-malate addition to the medium reservoir on L-malate accumulation, lactate production, and cell yield of glucose-limited continuous culture (8 mM glucose, 0.1 h^{-1}, pH 4.5). (b) Relationship between L-malate addition and the L-malate utilization rate.

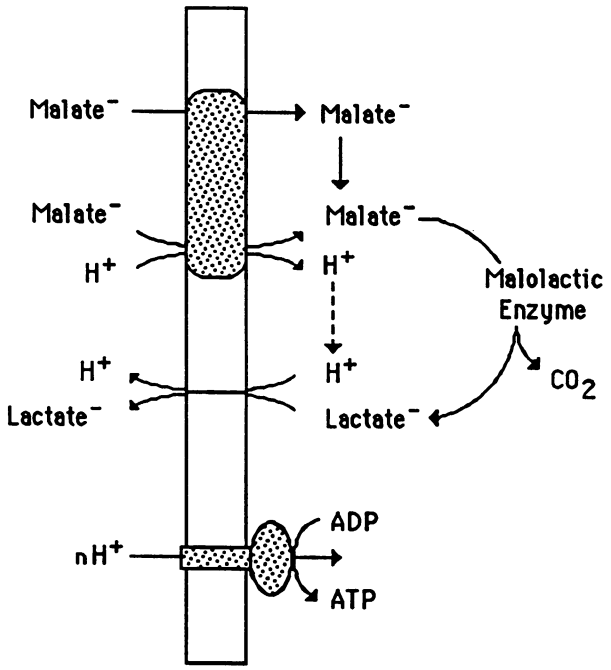

FIG. 11. Hypothetical scheme of malate-dependent energy derivation by *L. plantarum*. At low malate concentrations, malate is transported by proton symport and electroneutral lactate efflux cannot create a $\Delta\Psi$. If the malate concentration is high, malate can enter the cell by a diffusion mechanism and electroneutral lactate efflux creates an electrochemical gradient of protons (Δp). The Δp can then generate ATP, and the yield of glucose-limited cells is then increased.

concentrations of malate were unable to generate as large a $\Delta\Psi$ (Fig. 9b).

Effect of L-malate on cell yield in continuous culture. When *L. plantarum* was grown in a glucose-limited chemostat (0.1 h^{-1}) at pH 4.5, more than 96% of the glucose was converted to an equal mixture of D- and L-lactate. The addition of L-malate to the medium reservoir caused a proportional increase in lactate, but there was initially little effect on the cell yield (Fig. 10a). Since virtually all of the malate was fermented, the utilization rate was proportional to the malate concentration (Fig. 11b). When the malate in the medium reservoir was increased from 20 to 75 mM, the rate of malate utilization exceeded the V_{max} of the high-affinity L-malate transport system (Fig. 8) and malate accumulated in the culture vessel (Fig. 10a). As malate accumulated, there was a gradual increase in cell yield. When the continuous culture was switched back to a medium containing only glucose, the cell yield decreased to its initial value. This latter observation indicated that the cells had not simply adapted to glucose limitation.

DISCUSSION

Recently isolated mutants of *Lactococcus lactis* and *Leuconostoc oenos* that possessed the malolactic enzyme were unable to ferment malate (38, 45). Thus, malic acid cannot readily diffuse across the cell membrane. In *E. coli* and *Salmonella typhimurium*, L-malate is transported by an inducible C_4-dicarboxylate permease that is catabolite repressed by glucose (25, 26). The L-malate transport system of *L. plantarum* was induced by L-malate, but it was not repressed by glucose. Henick-Kling (21) noted that the malolactic specific activity of *L. plantarum* was greatest at pH 3.5, and we observed maximal transport activity when the cells were grown at this pH.

Recent work with the ruminal bacterium *Oxalobacter formigenes* showed that oxalate was transported via an anion-exchange mechanisms involving formate (2). Because malate transport could be driven by a lactate gradient, the possibility of malate-lactate exchange was initially attractive. However, subsequent experiments provided little evidence for an exchange mechanism: (i) high-affinity malate transport could not be driven by a lactate gradient in the absence of a Δp (Fig. 4b), and (ii) an artificial $Z\Delta pH$ created by acetate diffusion drove high-affinity malate transport in the absence of lactate (Fig. 4a). Based on these observations, it appeared that lactate efflux created a ΔpH that subsequently drove malate uptake. Proton symport mechanisms for malate transport have also been observed in *Saccharomyces cerevisiae*, *E. coli*, and *Klebsiella aerogenes* (6, 13, 17).

Harold et al. (19) showed that lactate translocation in *E. faecalis* could be driven by a ΔpH (inside alkaline) and suggested that lactate translocation across the cell membrane was mediated by a carrier (18). Lactate uptake by *L. plantarum* was also ΔpH dependent (Fig. 5a), but there was little evidence that lactate translocation was carrier mediated. Counterflow, a common characteristic of carrier-mediated transport, could not be demonstrated in the absence of a Δp (Fig. 5b). *S. cerevisiae* has an inducible lactate-proton carrier, but undissociated lactate was also taken up by a noninducible diffusion mechanism (5).

In 1979, Michels et al. (30) proposed that carrier-mediated lactate efflux could generate a Δp (lactate/proton ratio of >1.0). This hypothesis was supported by the following observations: (i) lactate efflux created a ΔΨ, (ii) an artificial ΔΨ drove lactate uptake, and (iii) increases in external lactate caused a decrease in growth rate and yield (32). In this energy recycling model, the lactate/proton stoichiometric ratio is only greater than 1 when the external pH is greater than 6.0 and external lactate is less than 20 mM (42, 43). Electrogenic lactate efflux was previously used as an explanation for malate-dependent ATP generation in *L. plantarum* (7, 8), but the present work does not support this hypothesis. (i) Lactate efflux from *L. plantarum* was unable to generate a ΔΨ at pH 4.5 or 6.5 even if the external lactate concentration was 1 mM, (ii) an artificial ΔΨ was unable to drive lactate uptake, and (iii) the greatest enhancements in cell yield were observed when the external lactate concentration was greater than 40 mM (Fig. 10a).

When *L. plantarum* was grown in a glucose-limited chemostat, malate addition caused as much as a 26% increase in cell yield (Fig. 10a). Because the addition of malate to de-energized cells caused a large increase in the ΔΨ and a subsequent increase in the ZΔpH (Fig. 9a), it appeared that a chemiosmotic mechanism was responsible for malate-dependent energy derivation. However, the energetics of this chemiosmotic mechanism were initially perplexing. Malate transport appeared to require a proton gradient, but lactate-proton efflux was electroneutral rather than electrogenic. If malate-proton symport was simply balanced by lactate-proton efflux, how could the cells benefit from the malolactic fermentation?

High-affinity malate transport required a ZΔpH and could be inhibited by TCS or iodoacetate, but the kinetics of malate transport were biphasic (Fig. 8). When malate was present at high concentrations (mM), TCS and iodoacetate had much less effect on malate fermentation (Fig. 1), and it appeared that malate was also taken up by a facilitated diffusion mechanism that did not require a ZΔpH. Because uninduced cells did transport malate even at high malate concentrations, the kinetics could not be explained by simple diffusion. Low-affinity lactate transport had a K_t that was similar to the K_m of the malolactate enzyme (15 mM versus 8 mM [40]).

The facilitated diffusion mechanism appeared to be an electrogenic process that involved malate^{-1}: (i) at low pH, the cells had little ΔΨ and malate was transported and fermented at a rapid rate (Fig. 2); (ii) when the pH was near neutral, the ΔΨ was large and the rates of malate transport and fermentation were lower (Fig. 2); and (iii) TPP^+, a lipophilic cation that would dissipate (ΔΨ), increased the rate of malate transport (Fig. 7). At this time it is not known whether the same carrier is responsible for low- and high-affinity malate uptake, but it should be noted that other Δp-driven carriers exhibited facilitated diffusion (36, 43).

Based on the observation that malate only caused an increase in Δp or cell yield when the external malate concentration was greater than 5 mM (Fig. 9b and 10a), the following mechanism seems likely (Fig. 11). When the malate concentration is less than 5 mM, most of the malate is taken up by proton symport, the balance of malate transport and lactate efflux is not electrogenic, a Δp is not created, and the malolactic fermentation provides little if any energy. As malate concentrations increase, the malate gradient serves as a driving force for transport (facilitated diffusion), the stoichiometry of malate-proton symport increases, the malate transport-lactate efflux couple becomes electrogenic, a decrease in intracellular proton concentration creates a Δp, and the Δp drives ATP synthesis. Malate-dependent ATP synthesis has been previously documented (7).

The energetics of malate conversion to lactate are not entirely straightforward. Thauer et al. (44) indicated that the standard free energy change ($\Delta G^{\circ\prime}$) of malate conversion to pyryvate and bicarbonate was +5 kcal (ca. +21 kJ)/mol and the $\Delta G^{\circ\prime}$ of pyruvate and bicarbonate conversion to lactate was −10.3 kcal (ca. −43.1 kJ)/mol. Since the free energy change should be independent of the route, the $\Delta G^{\circ\prime}$ of the malolactic reaction should be −5 kcal (ca. −21 kJ)/mol. Under physiological conditions, however, malate, lactate, and bicarbonate concentrations are less than 1 M. As the malate concentration decreases and the lactate and carbon dioxide concentrations increase, there would be less $\Delta G'$ to drive ATP formation ($\Delta G' = \Delta G^{\circ\prime} + 1.36 \log([\text{lactate}^{-1}][HCO_3^{-1}]/[\text{malate}^{-2}])$. At the midpoint of the reaction (lactate and malate at equal concentrations), $\Delta G'$ depends on the concentration of bicarbonate. The estimation of a true $\Delta G'$ is further complicated by the effect of pH on bicarbonate concentration. As the pH declines, bicarbonate declines and the $\Delta G'$ would increase.

L. plantarum is a bacterium that has adapted to growth in a highly acidic environment, and this pH resistance has been manifested in a variety of ways. Like many other lactobacilli (24), *L. plantarum* allows its intracellular pH to decrease as a function of extracellular pH. At pH 4.5, (i) the malolactic fermentation occurs at its maximum rate, (ii) the intracellular pH corresponds with the pH optimum (6.0) of the malolactic enzyme (20), (iii) the specific activity of the malate carrier increases, (iv) malate^{-1} predominates, (v) there is little ΔΨ, and (vi) malate is taken up by a mechanism that does not require ΔΨ or is in some cases inhibited by ΔΨ.

The relative contribution of malate^{-1}-proton versus malate^{-1} transport is not entirely clear, but both systems may operate at high substrate concentrations. TCS-treated cells were able to ferment malate, but the fermentation rate was twofold greater when a Δp was present (Fig. 1). The cell yield of glucose-limited continuous cultures was increased by the addition of malate, but the stimulation was only 26% even though the rate of malate fermentation was greater than 500 nmol/min/mg of protein (Fig. 10). If all of the malate had been taken up as malate^{-1} and the stoichiometry of the membrane-bound ATPase was 3 protons per ATP molecule, one would have expected a much larger increase in the cell yield (Fig. 11).

Recent work has indicated that bacteria are able to derive energy from organic acid fermentations that have only a small change in free energy. *Propionigenium modestum* has a membrane-bound decarboxylase that is able to translocate sodium outwardly through the cell membrane, and the sodium gradient then drives ATP synthesis (12). In *O. formigenes*, oxalate^{-2} is taken up via an exchange involving formate^{-1}, and proton utilization creates a Δp, which then drives ATP formation (2). The malate^{-1} transport and lactate^{-1}-proton efflux system of *L. plantarum* represents yet another pattern of bacterial energy conservation.

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