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Electrogenic Malate Uptake and Improved Growth Energetics of the Malolactic Bacterium *Leuconostoc oenos* Grown on Glucose-Malate Mixtures

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Growth of the malolactic bacterium *Leuconostoc oenos* was improved with respect to both the specific growth rate and the biomass yield during the fermentation of glucose-malate mixtures as compared with those in media lacking malate. Such a finding indicates that the malolactic reaction contributed to the energy budget of the bacterium, suggesting that growth is energy limited in the absence of malate. An energetic yield (Y_{ATP}) of 9.5 g of biomass · mol ATP^{-1} was found during growth on glucose with an ATP production by substrate-level phosphorylation of 1.2 mol of ATP · mol of $glucose^{-1}$. During the period of mixed-substrate catabolism, an apparent Y_{ATP} of 17.7 was observed, indicating a mixotrophy-associated ATP production of 2.2 mol of ATP · mol of $glucose^{-1}$, or more correctly an energy gain of 0.28 mol of ATP · mol of $malate^{-1}$, representing proton translocation flux from the cytoplasm to the exterior of 0.56 or 0.84 H^+ · mol of $malate^{-1}$ (depending on the H^+/ATP stoichiometry). The growth-stimulating effect of malate was attributed to chemiosmotic transport mechanisms rather than proton consumption by the malolactic enzyme. Lactate efflux was by electroneutral $lactate^-/H^+$ symport having a constant stoichiometry, while malate uptake was predominantly by a $malate^-/H^+$ symport, though a low-affinity $malate^-$ uniport was also implicated. The measured electrical component ($\Delta\psi$) of the proton motive force was altered, passing from −30 to −60 mV because of this translocation of dissociated organic acids when malolactic fermentation occurred.

Certain species of lactic acid bacteria (belonging to the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc*) possess the capacity to convert malate to lactate via a direct decarboxylation reaction referred to as malolactic fermentation. This fermentation is of use in the enological industry since it enables the removal of excess acidity, enhances organoleptic properties, and increases the bacteriological stability of wine (7, 33).

Those bacteria able to metabolize malate via malolactic fermentation show improved growth characteristics when presented with glucose-malate mixtures as compared with the growth characteristics during fermentation of glucose alone. This response to an auxiliary substrate is not, at first glance, surprising: increased growth yields attributable to a second catabolic substrate have been previously reported (2, 13). In these reports the maximum specific growth rates remained unaltered, being fixed by carbon flux limitations. More recently, improved growth yields and specific growth rates have been achieved with substrate mixtures which overcome both carbon flux and energetic limitations simultaneously (15). In the case of the malolactic bacteria, the situation is somewhat different in that the malate cannot contribute directly to the anabolic carbon flux and, furthermore, there is no energy conservation at the enzyme level (either directly by substrate-level phosphorylation [SLP] or indirectly via reducing equivalent generation). The reaction involved in the decarboxylation of malate is catalyzed by a single enzyme, the malolactic enzyme (EC 1.1.1.38), which transforms malate to lactate and CO_2 without release of pyruvate (3). Stimulated utilization of carbon substrates has been suggested to explain the improved growth, as has an ecological function enabling a favorable pH to be maintained. This second explanation has been shown to be false since the growth effect was also obtained with growth under constant pH conditions (22, 26). Recently, it has been demonstrated that ATP formation results from the malolactic fermentation (5, 6, 23). Since SLP reactions can be ruled out and direct ion extrusion involving membrane-bound decarboxylase(s) (8) does not occur, the generation of biochemical energy via substrate uptake or product efflux has been examined. One such possibility would be the energy recycling model of lactate efflux (24, 29), though recently alternative explanations have been proposed. The malolactic fermentation of *Lactococcus lactis* generates metabolic energy via a malate-lactate antiport and electrogenic malate uptake in combination with efflux diffusion of lactate and proton consumption during the decarboxylation reaction (23). The net energy gain would be equivalent to a proton translocated from the cytoplasm to the broth per malate molecule consumed. Such a model is somewhat similar to that proposed for *Oxalobacter formigenes* and the oxalate-formate exchange (1). A different mechanism has been proposed for the gain in energy by the malolactic fermentation of *Lactobacillus plantarum* in which ATP is also produced via the intermediate of the proton motive force (PMF) (20). This model differs in that malate uptake involves uniport translocation of the $malate^-$ ion rather than the antiport mechanism.

In this paper the growth-stimulating effect of malate on *Leuconostoc oenos* was investigated first to characterize the real growth improvements attributable to malate metabolism

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and second to ascertain which energetic mechanism was implicated during the phase in which growth stimulation could be quantitatively demonstrated.

MATERIALS AND METHODS

Organism, culture medium, inoculum preparation, and culture conditions. The *L. oenos* strain used in this study was supplied by RP-TEXEL, Dangé Saint-Romain, France, and was isolated from a Burgundy red wine. The standard culture medium had the following composition: tryptone, 20 g · liter^{-1}; yeast extract, 5 g · liter^{-1}; K_2HPO_4, 2 g · liter^{-1}; $MgSO_4$, 0.2 g · liter^{-1}; $MnSO_4$, 0.5 g · liter^{-1}; glucose, 25 g · liter^{-1}; and L-malic acid, 10 g · liter^{-1}. The pH was adjusted to 5.0 with NaOH (10 M).

Lyophilized cells, stored at −20°C, were reactivated by cultivation on this medium in screw-cap culture test tubes for 5 days at 27°C.

Batch culture experiments were performed in 2-liter fermentors at a temperature of 27°C and an agitation speed of 200 rpm. Cultures were carried out with pH control at a constant value (pH 5.0) by automatic addition of orthophosphoric acid (1.6 M) or ammonium hydroxide (10 M). The medium was gassed with N_2 immediately prior to inoculation. The inoculum was cells in the late growth phase.

Fermentation kinetic analysis. Bacterial growth was measured turbidimetrically at 660 nm and calibrated to cell dry weight as previously described (26). The specific growth rates were calculated from the cell mass profiles.

Concentrations of substrates (glucose and malic acid) and products (ethanol and acetic and lactic acids) in the fermentation broth were measured by high-pressure liquid chromatography (HPLC) using an ion exclusion column as previously described (26). L-Lactic acid was measured by an enzymatic method with a YSI model 2000 analyzer. The amounts of D-lactic acid were expressed as the difference between total lactic acid (HPLC measurement) and L-lactic acid (YSI determination) concentrations.

Measurement of ΔpH and Δψ. The cross-membrane gradients of pH (ΔpH) and electrical component (Δψ) were assayed by measuring the accumulation of [^{14}C]benzoate and ^{3}H-tetraphenylphosphonium bromide ([^{3}H]TPP$^+$), respectively (12, 25), by the silicon oil centrifugation technique (28). Portions of 1.3-ml samples were incubated for 3 min at 27°C with 15 μM [carboxyl · ^{14}C]benzoate (15 mCi · mmol^{-1}) or 10 μM [^{3}H]TPP$^+$ (23 mCi · mmol^{-1}) and transferred to microcentrifuge tubes containing silicon oil (0.2 ml of 1.02-g · ml^{-1} density). The cells were separated from the external medium by centrifugation at 17,500 × *g* for 15 min at room temperature. Supernatant fractions were taken and transferred into vials containing 2 ml of Ready Gel scintillation fluid, and the radioactivity content was determined by using a Beckman LS1801 scintillation counter. The remaining supernatant and the oil fraction were removed by suction, and the walls of the centrifuge tube were thoroughly wiped clean. NaOH (500 μl of 0.5 M) was added to the cell pellet. After homogenization, the suspension was incubated at room temperature for 30 min and transferred into counting vials containing 5 ml of scintillation cocktail. Radioactivity was determined as described above. The intracellular benzoate and TPP$^+$ concentrations were calculated with the intracellular volume obtained as described below.

However, before [^{3}H]TPP$^+$ accumulation was calculated, the amount of [^{3}H]TPP$^+$ bound to the cells had to be subtracted from the total amount of [^{3}H]TPP$^+$ found in the pellet. After pretreatment of the cellular suspension with 10 μM carbonyl cyanide chlorophenylhydrazone, TPP$^+$ binding was measured as described above. Other controls, using 0.1 μM nigericin, led to a total abolition of ΔpH, showing that there was no significant adhesion of [^{14}C]benzoate to the cell debris. Further controls, i.e., toluene (2%)- or butanol (10%)-treated cells, were used to collapse the PMF, confirming results with controls described above, but resulted in experimental difficulties regarding the centrifugation through oil and were not systematically employed.

Measurement of intracellular volume. Samples (1.3 ml) from the fermentors were incubated with 4 μCi of ^3H_2O (5 mCi · ml^{-1}) and 1.6 μM ^{14}C-polyethylene glycol 4000 (60 mCi · mmol^{-1}) and handled as described above. ^3H_2O was used to label the total water volume of the pellet, and ^{14}C-polyethylene glycol 4000 was used to label the extracellular volume.

The internal cell volume did not vary significantly during the different stages of growth and was 2.09 ± 0.16 μl · mg^{-1} (dry matter).

From the data on intracellular and extracellular water content and the amount of radioactivity in supernatant and pellet fraction, the accumulation ratios of [^{14}C]benzoate and [^{3}H]TPP$^+$ were calculated.

Measurement of internal organic acid concentrations. Portions (0.9 ml) taken directly from the cultures were transferred into microcentrifuge tubes containing 0.4 ml of silicon oil on top of 0.2 ml of 22% (wt/wt) perchloric acid solution (density, 1.14 g · ml^{-1}) and centrifuged at 17,500 × *g* for 15 min at ambient temperature. Thus, biomass was separated from the supernatant, and in perchloric acid, rapid disruption of the cells and inhibition of further metabolism occurred. The L- and D-lactate and L-malate concentrations in supernatant and in the perchloric acid fraction were determined enzymatically with reagents and methods from Boehringer, Mannheim, Germany. Intracellular lactate and malate concentrations were also measured in each fraction by the same enzyme-coupled spectrophotometric analysis techniques and took into account the intracellular water concentration.

RESULTS AND DISCUSSION

Fermentation products during growth on glucose or glucose-malate mixtures. The substrate consumption and product formation profiles for a culture of *L. oenos* growing in medium containing glucose (25 g · liter^{-1}) and malate (10 g · liter^{-1}) are shown in Fig. 1. Glucose and malate were consumed simultaneously; malate was transformed exclusively via the malolactic reaction to CO_2 and L-lactate, while glucose was transformed with constant stoichiometry to mixtures of D-lactate, acetate, ethanol, and CO_2 with yields of 1.0, 0.18, 0.68, and 1.0 mol · mol^{-1}, respectively, throughout the fermentation. These data are in agreement with the theoretical values normally associated with the malolactic reaction and the phosphogluconate pathway of glucose catabolism (11). However, a closer look at the stoichiometry shows that an imbalance occurred for both major elements and redox equivalents. For carbon, hydrogen, oxygen, and redox balance, recovery percentages of 95, 80, 100, and 85%, respectively, were obtained. A similar imbalance occurred for the cultures lacking malate. While the possibility of some incorporation of glucose into biomass cannot be excluded, an unidentified compound was observed during chromatographic analysis of the fermentation broth. Furthermore, this compound was produced in direct proportion to the glucose consumed, though attempts to identify it

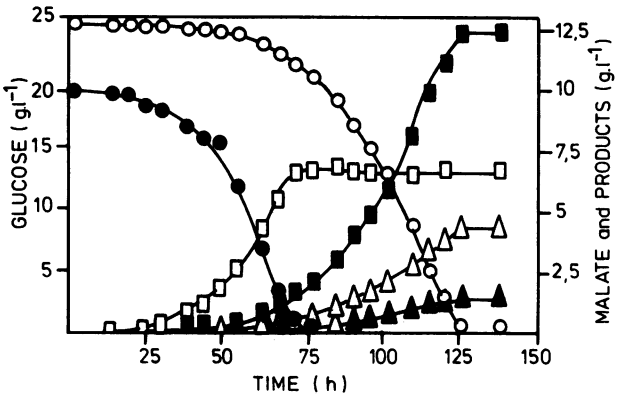

FIG. 1. Batch fermentation profile for the growth of *L. oenos* on glucose (25 g · liter^{-1}) plus malate (10 g · liter^{-1}) at pH 5.0. ○, Glucose; ●, malate; □, L-lactate; ■, D-lactate; △, ethanol; ▲, acetate.

have not been successful. In view of the product distribution pattern and the metabolic constraints, this unknown compound would have been derived from acetyl coenzymeA but did not cochromatograph with acetoacetate, acetone, diacetyl, acetaldehyde, butyrate, pyruvate, or intermediates of either the tricarboxylic acid cycle or the glyoxylate bypass.

A similar fermentation profile was seen for the control experiment lacking malate (results not shown), except that L-lactate was not produced and the fermentation took somewhat longer, 170 h rather than 100 h.

Growth-stimulating effect of the malolactic fermentation. As previously reported (26), enhanced growth rates were obtained when malate was used in addition to glucose. Maximum growth rates corresponding to the early stages of growth were 0.082 and 0.052 h^{-1} for growth on glucose-malate and glucose alone, respectively. In addition to this stimulation of the growth rate, malate consumption also led to improved biomass yields. A plot of biomass production against glucose consumption (Fig. 2) shows that two clearly distinct phases occurred during glucose-malate fermentation. The first phase corresponded to the double-substrate consumption phase, while the second represents that part of the fermentation which occurred after depletion of the malate, i.e., growth on the remaining glucose. Apparent

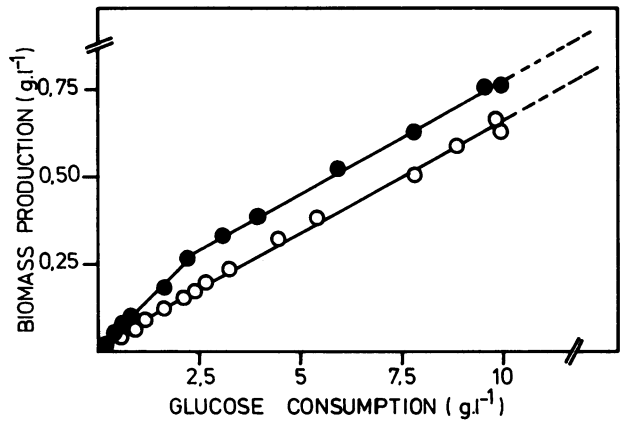

FIG. 2. Biomass production versus glucose consumption for the fermentation of *L. oenos* on glucose-malate (●) or glucose alone (○).

biomass yields related to glucose, calculated as the gradient of the plot, were significantly higher during the mixed-substrate growth phase than in the phase of growth which followed. In this second phase, the apparent growth yield was similar to that calculated during fermentations which lacked malate. This effect can be taken as clear evidence that there was an energy gain associated with malolactic fermentation. Similar results were obtained by Cogan (4) for the growth of *Leuconostoc* spp. on glucose-citrate mixtures, although the potential gain in energy from the catabolism of citrate is easily explained, unlike the present situation with glucose-malate mixtures in which no such direct gain in biochemical energy can be identified. The current opinion is that the biochemical energy associated with the malolactic reaction is not generated either by substrate-level phosphorylation reactions or by net reducing-equivalent gains. The complete recovery of the malate as lactate and CO_2 also precludes any role as regards a biosynthetic precursor donor. It is therefore necessary to determine exactly how the decarboxylation of malate to lactate can generate useful metabolic energy.

Energetic aspects of the growth of *L. oenos*. In order to elucidate this growth-stimulating phenomenon, kinetic evidence was sought by first calculating the known rates of energy supply (SLP reactions) and utilization and, second, measuring the transmembrane gradients (ionic charge, protons, and acids) to assess any electrochemical consequences of malate consumption.

Taking into account the heterofermentative pathway of glucose metabolism in *Leuconostoc* species, the net gain in SLP is 1 mol of ATP · mol of D-lactate^{-1} and 1 mol of ATP · mol of acetate^{-1}. Since the organic acid conversion yields obtained throughout growth on glucose remained constant, the SLP-associated production of ATP was 1.18 mol of ATP · mol of glucose consumed^{-1}. The biomass synthesis associated uniquely with glucose consumption (i.e., during growth on glucose alone) was 11.2 g of biomass · mol of glucose^{-1}, enabling an ATP yield (Y_{ATP}) of 9.5 g of biomass · mol of ATP^{-1} to be estimated. Similar calculations for the mixotrophic phase of the glucose-malate fermentation, for which the biomass yield related to glucose consumption was 20.9 g of biomass · mol of glucose^{-1}, indicates an apparent Y_{ATP} of 17.7 g of biomass · mol of ATP^{-1}. During the second phase of this fermentation, a Y_{ATP} similar to that associated with growth on glucose (in the absence of malate) was observed. The Y_{ATP} of 9.5 should therefore be taken as a correct average value of the energetic efficiency of growth on glucose. Recalculating the ATP balance during the mixotrophic growth phase with this Y_{ATP} value indicates that a true ATP formation of 2.2 mol of ATP · mol of glucose^{-1} was necessary for biomass formation. Since only 1.2 mol of ATP can be accounted for by SLP reactions directly associated with glucose metabolism, further ATP must be produced by other mechanisms specifically linked to malate consumption. It is more reasonable to express this energy gain relative to the malate consumption. During the period of mixed-substrate consumption, the overall molar substrate consumption ratio was 3.5 mol of malate · mol of glucose^{-1}, enabling the ATP gain directly associated with malate to be estimated as 0.28 mol of ATP · mol of malate^{-1}. If the energy gain were due to a chemiosmotic mechanism linked to malate uptake or lactate efflux, the number of protons translocated from the interior to the exterior of the cell can be estimated to have been 0.56 or 0.84 H^+proton · mol of malate^{-1} for H^+/ATP stoichiometries of ATPase equal to 2 or 3, respectively. It remains

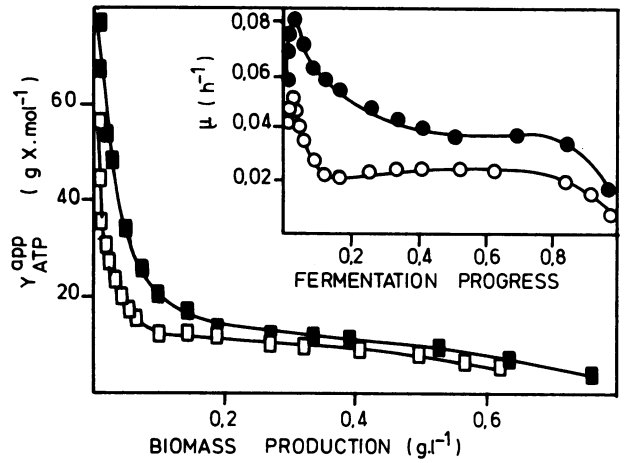

FIG. 3. Profiles of apparent Y_{ATP} values calculated during growth on glucose-malate (■) or glucose alone (□). (Insert) Evolution of specific growth rates on glucose-malate (●) or glucose alone (○) as a function of fermentation progress relative to biomass formation ($X - X_0/X_m - X_0$, where X is actual biomass concentration, X_0 is initial biomass concentration, and X_m is maximal biomass concentration).

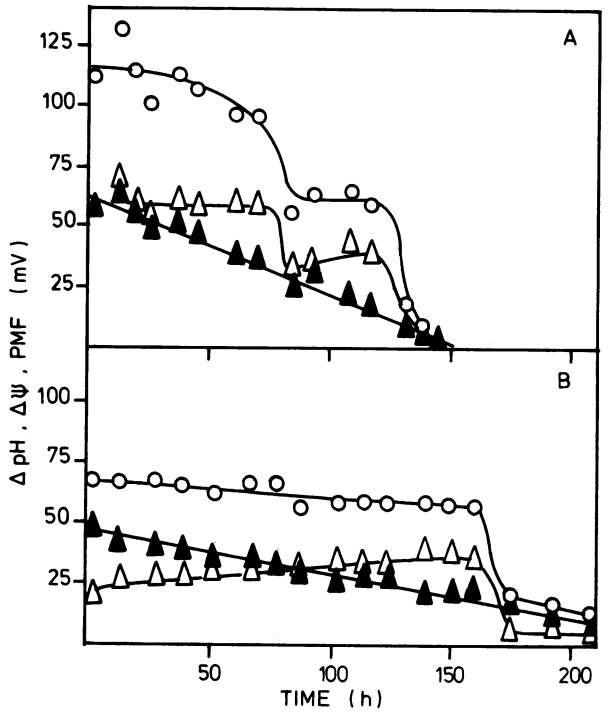

FIG. 4. Evolution of PMF (○) and its components, ΔpH (▲) and Δψ (△), during batch-mode fermentation of *L. oenos* on glucose plus malate (malate exhausted at 75 h) (A) and glucose alone (B). The values given for Δψ and PMF should be read as negative values.

essential to measure organic acid transmembrane concentration gradients and the various components of the PMF to substantiate the possible involvement of a chemiosmotic mechanism.

The values discussed above are global values representing whole fermentations or phases of growth. When the dynamic profile of apparent Y_{ATP} values was examined as a function of the fermentations' progress relative to biomass formation, extremely high values were obtained at the beginning of the culture irrespective of the presence or absence of malate and, furthermore, growth rates were significantly higher during this period (Fig. 3). These Y_{ATP} values in excess of 50 g of biomass · mol of ATP^{-1} can be explained only if an alternative form of energy production existed, though no obvious explanation can be advanced. The most probable explanation of this finding would be the consumption of trace amounts of oxygen during the early stages of growth and respiratory production of ATP, as has been suggested previously for *Leuconostoc mesenteroides* (16). Control experiments showed a dissolved oxygen content of zero immediately after N_2 gassing, but an increase in partial O_2 pressure (measured with a dissolved oxygen probe) which would be sufficient to enable some respiratory activity was seen in noninoculated fermentors. Furthermore, the similar profile obtained for the two fermentations as regards apparent Y_{ATP} evolution does not invalidate the energetic comparisons, since medium preparation and fermentation vessels were standardized.

PMF variations during growth. The time course of the PMF and of its individual components (Δψ and ΔpH) measured throughout the fermentation of *L. oenos* on glucose or glucose-malate mixture standard medium at pH 5.0 is shown in Fig. 4.

Maximal ΔpH was obtained at the beginning of the growth and decreased progressively throughout fermentation irrespective of the substrate(s) used. Since external pH was kept constant, this dissipation of the ΔpH represents an acidification of the cytoplasm related certainly to the production of organic acids (acetic and lactic acids). The rate at which the collapse of the ΔpH occurred seems to have been directly related to the concentration of acid produced: the fall in ΔpH was larger for growth on glucose-malate mixtures than for growth on glucose alone because of higher rates of production of lactic acid. When cultivated at a lower pH value (pH 4.0), the organism responded by a more rapid fall in ΔpH (results not shown), as would be expected if the dissipation of this component of the PMF were due to a decoupling effect of the free acids (32).

It is important to note that malate exhaustion was without effect on the ΔpH profile and that the ΔpH measured under mixotrophic growth conditions was only slightly higher than that on glucose alone. This is in contrast to the clear-cut effect of malate on the Δψ. In the absence of malate, a progressive increase was seen in the Δψ throughout the fermentation to partially compensate for the fall in ΔpH and thereby keep the PMF almost constant. The addition of malate to glucose-grown cells after approximately 30 h of cultivation resulted in a rapid change in the Δψ from a value of −27 to −56 mV, a value maintained until malate had been entirely consumed: the Δψ then reestablished at −30 mV upon a return to metabolism on glucose alone (results not shown). When the Δψ profile was examined in cells grown on glucose-malate mixtures, this same change was observed during the period of coconsumption, with a value of −60 mV maintained up until exhaustion of the malate, at which point the Δψ fell to −35 mV. This increased Δψ component of the PMF was not compensated for by a decreased ΔpH but gave rise to a greater overall PMF: −110 mV during glucose-malate coconsumption and −60 mV during consumption of glucose alone (either in the second phase of the mixed-substrate fermentation or in control fermentations). The complete and rapid dissipation of both components of the PMF was observed in all cases when catabolic substrates

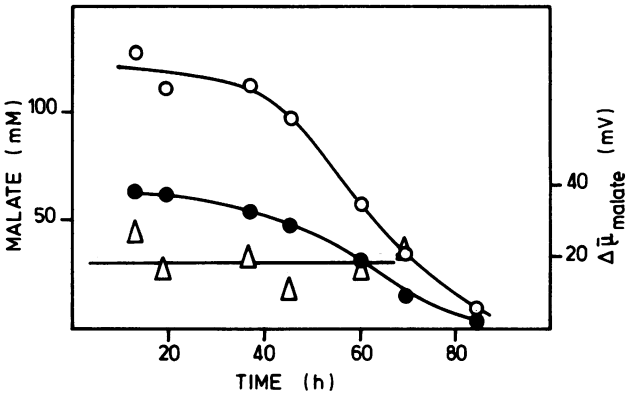

FIG. 5. Measured intracellular (○) and extracellular (●) malate concentrations and electrochemical gradient (Δμ̄) of malate (△) during growth on glucose-malate.

were no longer present. The addition of the nonmetabolizable D-malate to the glucose-grown cells during the active growth phase had no effect on the membrane potential, showing that the increased membrane potential described above resulted from the active metabolism of the L-malate and was not a passive response of the cells to the organic acid. These observations indicate that the energy gain obtained from the malolactic fermentation is most probably linked to the generation of a transmembrane electrochemical proton gradient.

Transmembrane organic acid gradients. Measurements of the intracellular concentrations of malate and lactate shed further light on the mechanism involved. Both the internal and the broth malate concentrations decreased throughout the fermentation such that the malate gradient was kept constant at approximately twice as much cytoplasmic malate as in the medium (Fig. 5). This gradient corresponds to an electrochemical charge gradient (Δμ̄) of 19 mV, a value significantly lower than the gradient which might be expected from the distribution of the ΔpH probe, benzoate, indicating that the constant malate equilibrium was never a simple function of the pH gradient.

The broth concentration of L-lactate increased during the fermentation as malate was consumed (Fig. 6). In the early stages of growth the intracellular concentration increased to levels slightly higher than might be expected from the pH

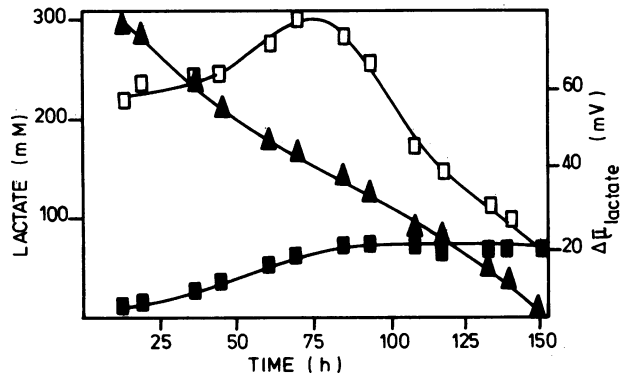

FIG. 6. Measured intracellular (□) and extracellular (■) L-lactate concentrations and electrochemical gradient (Δμ̄) of L-lactate (▲) during growth on glucose-malate.

gradient. Once the malate had been consumed, the L-lactate gradient rapidly equilibrated with that predicted from ΔpH considerations. Similar results were obtained for the D-lactate gradient (data not shown). These results can be used to assess the potential implication of various forms of transport for both the substrate and the product.

Energetics of organic ion transporters. If it is assumed that the organic acid gradient is at all times close to its thermodynamic equilibrium (as postulated by Ten Brink and Konings [28]), the number of protons translocated in symport with each molecule of acid can be calculated. Indeed, the driving force for carrier-mediated solute translocation in symport with protons can be given by the following equations from the chemiosmotic mechanism proposed by Mitchell (18, 19): for inside-to-outside translocation of lactate, driving force = $\Delta\bar{\mu}_A + (m + n) \cdot \Delta\psi - nz \cdot \Delta pH$ (in mV), and for outside-to-inside translocation of malate, driving force = $-\Delta\bar{\mu}_A - (m + n) \cdot \Delta\psi + nz \cdot \Delta pH$ (in mV), in which $\Delta\bar{\mu}_A = z \cdot \log(A_{in}/A_{out})$ is the chemical gradient of solute A m is the charge of the solute; n is the number of protons symported; A_{in} and A_{out} are the internal and external concentrations of the solute, and z is equal to 2.3 RT/F, where R is the perfect gas constant, T is temperature (in kelvins), and F is Faraday's constant.

For each equation, a steady state is reached when the driving force is equal to zero or when $\Delta\bar{\mu}_A = -m\Delta\psi - n$PMF. Since at the experimental pH (pH 5.0), charge values (m) of lactate and malate calculated from established pK values were −1.00 and −1.32, respectively, the number of protons symported (n) can be calculated from the following equations: $n = (\Delta\psi - \Delta\bar{\mu}_{lactate})$/PMF and $n = (1.32\Delta\psi - \Delta\bar{\mu}_{malate})$/PMF.

Lactate efflux. For L-lactate efflux, the n values calculated throughout the fermentation were always close to 1. The same constant value of 1 was also found for D-lactate and hence for total lactate. These data indicate a constant H^+/lactate stoichiometry of 1, independent of lactate concentration or gradient or of ΔpH values. This constant stoichiometry seems to be a common feature of the malolactic bacteria studied to date: similar results have recently been published for *L. plantarum* (20) and *L. lactis* (23). The only energetic contribution of such an electroneutral symport can be the maintenance of a ΔpH. Lactate efflux did not contribute to a Δψ increase, confirming the findings of Olsen and coworkers (20). The energy recycling model associated with variable stoichiometry efflux of lactate as proposed by Konings' group (17, 28) was clearly not operating during growth of *L. oenos*.

Since the energetic consequences of this symport system are identical with or without carrier-mediated translocation, it is difficult to differentiate passive and facilitated diffusion. While evidence for carrier-mediated transport has been presented for many acidogenic bacteria, including *Streptococcus faecalis* (10), *Streptococcus cremoris* (21, 28, 29), *Lactobacillus helveticus* (9), *L. plantarum* (30), and *Pseudomonas mendocina* (31), it is all the more surprising that recent results by Olsen et al. (20) indicate that lactate translocation was by passive diffusion alone during the malolactic fermentation of *L. plantarum*. For *L. lactis* a lactate-malate antiport (23) somewhat similar to the oxalate-formate exchange of *O. formigenes* (1) was accompanied by a carrier-mediated or passive efflux of lactic acid. Since n values were always equal to 1, no such antiport operates in *L. oenos*.

Malate uptake. Since the only energy gain obtained from lactate efflux was a contribution to ΔpH maintenance, the

growth-stimulating effect of malate must be attributed to a more important energy gain associated with malate uptake. Indeed, the *n* values calculated from the last equation in "Energetics of organic ion transporters," above, show a variable malate/H^+ stoichiometry. At the beginning of growth, when the malate concentration was highest (75 mM), *n* values of 0.8 were calculated, though values of 1.0 were achieved towards the end of the malate consumption phase, when the residual malate concentration fell below 16.5 mM, indicating that the energy gain was variable and directly linked to the malate concentration in the medium. The malate gradient remained constant during this period of variable stoichiometry (Fig. 5). Taking the average value of 0.88 for the malate consumption phase indicates that the contribution of a malate/H^+ symport to total malate uptake was 88%.

Two symport systems, a malate^{2-}/H^+ and a malate$^-$/H^+, could exist. These alternatives have different energetic consequences with respect to charge translocation and also due to a different evolution of each species in the cytoplasm. Taking into account an average internal pH of 5.75 during the malate consumption phase, the ratio of malate$^-$ to malate^{2-} changed from 63%/35% in the external medium to 24%/76% in the cytoplasm. Therefore, uptake of only malate$^-$ would impose an intracellular dissociation in accord with the following relationship: 1 malate$^-$ → 0.24 malate$^-$ + 0.76 malate^{2-} + 0.76 H^+. Inversely, the translocation of malate uniquely as the fully dissociated form would involve proton consumption: 1 malate^{2-} + 0.24 H^+ → 0.24 malate$^-$ + 0.76 malate^{2-}.

It is generally accepted that the decarboxylation of malate to lactate involves the consumption of an H^+ proton per malate, thus creating an energy gain, though in our view no evidence exists to support this hypothesis. Faced with the lack of evidence to support this proposed reaction—1 malate$^-$ + 1 H^+ → 1 lactic acid + 1 CO_2—several observations can be made: (i) as yet no evidence exists as regards the substrate specificity of the malolactic enzyme for one form or another of the malate, (ii) the optimal pH for maximum activity of the malolactic enzymes examined to date is close to the cytoplasmic pH under optimal conditions of growth on glucose-malate mixtures, i.e., 5.5 to 5.8 (3, 14, 27), and (iii) lactate$^-$ is the only form present at this cytoplasmic pH. Clearly, only if malate^{2-} is transformed to lactate$^-$ will a proton be consumed.

In view of these criteria the energy gain calculations presented below have been made assuming that the intracellular pH-dependent dissociation of malate was respected, giving the following stoichiometry for the malolactic reaction: 0.24 malate$^-$ + 0.76 malate^{2-} + 0.76 H^+ → 1 lactate$^-$ +1 CO_2.

In addition to the malate/H^+ symport, another electrogenic malate uptake system must operate to account for the 12% not transported by the symport(s). This second system was independent of protons, and two possible mechanisms can be envisaged: a malate^{2-}-lactate$^-$ antiport (23) and a uniport translocation of malate$^-$ (20). Both processes have the same energetic consequences, though the operation of the antiport would, bearing in mind the experimental data, necessitate a variable proton stoichiometry for the efflux of lactate (from 0.8 to 1.0). Since this was not the case, this possibility can be eliminated and this part of the malate uptake can be attributed to a uniport whose function has been shown to be dependent on the malate concentration (see above).

Energy flux models of the malolactic reaction. Taking into consideration existing models of the energetic aspects of malolactic fermentation and modifying these to include our own observations related to the presence of a malate uniport, the energy gain associated with two possible variants (Fig. 7) can be estimated in terms of proton equivalents translocated per malate consumed.

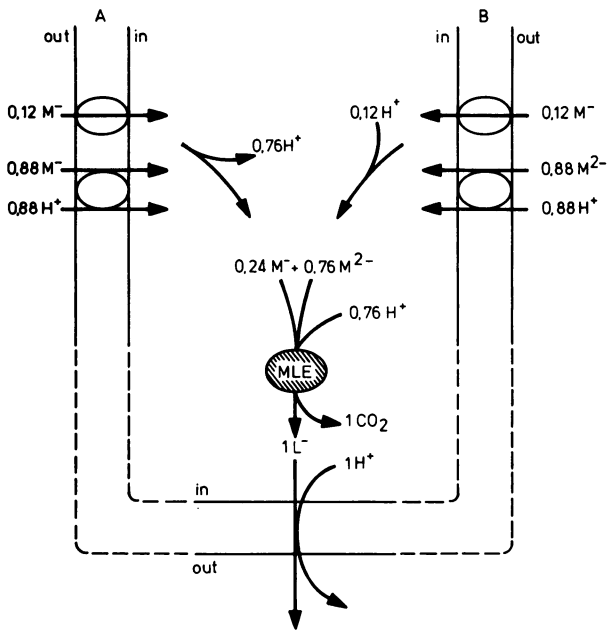

FIG. 7. Models of malolactic fermentation that may occur in *L. oenos* cells growing on glucose-malate at pH 5.0, including malate uptake by malate$^-$ uniport and either malate$^-$/H^+ symport (A) or malate^{2-}/H^+ symport (B); malate decarboxylation by the malolactic enzyme (MLE) to lactate and CO_2 and lactate$^-$/H^+ symport efflux. Dissociated acids: M^{2-}, malate^{2-}; M^-, malate$^-$; L^-, lactate$^-$.

The variant based on the malate$^-$/H^+ symport (Fig. 7A) predicts a net gain of 0.24 proton equivalent (0.12 H^+ proton and 0.12 A^- ion) per malate, with −0.88 H^+ proton + 0.12 A^- ion (negative charge) per malate transported and 1 H^+ proton per lactate exported. In this case no energy gain would occur at the decarboxylation step, since the proton movement was equilibrated.

For the malate^{2-}/H^+ symport variant (Fig. 7B), the net gain would be 2.0 proton equivalents (1.0 H^+ proton and 1.0 A^- ion) in which the following balances would be involved: 0.12 A^- ion associated with the malate$^-$ uniport, 0.88 A^- ion minus 0.88 H^+ proton for the malate symport, 1 H^+ proton associated with lactate efflux, and a total of 0.88 H^+ proton equivalent associated with the malolactic reaction.

If these predictions are placed in the context of the real gain in growth performance as indicated from biomass yield improvements and Y_{ATP} profiles, this second model undoubtedly produces far too much energy. It would seem unlikely that this symport is operating to any significant extent. The present research would tend to favor the malate$^-$/H^+ model, though the steady-state gradients measured here do not enable the totality of the necessary energy to be visualized. This low energy gain (0.24 rather than the predicted 0.56 or 0.84 H^+ proton equivalent based on ATP/H^+ stoichiometry of 2 or 3, respectively) can be explained if it is remembered that these experiments were undertaken with actively metabolizing cells in which dynamic equilibria exists among PMF, adenylate charge (or

ATP concentration), and anabolic reactions. Since the energy gain was immediately and continuously used for anabolic reactions, it would logically be underestimated by the procedure used here, which was based on steady-state concentration equilibria rather than true rates of energy production. The malate-dependent increase in the PMF measured in this study with actively growing cells was considerably lower than that measured for deenergized *L. plantarum* in which no such growth artifacts were involved (20). Furthermore, such an approach also enabled increases of both the ΔpH and the $\Delta\psi$ component of the PMF to be visualized. While our results concur with those of Olsen et al. (20), it cannot be definitively ruled out that the $malate^{2-}/H^+$ symport might play a role in the uptake of malate, though in the growth conditions employed here this would not be a major contribution.

Concluding remarks. In recent years a number of malolactic bacteria have been investigated, and it would appear that different species possess one or another of the symport mechanisms of malate transport discussed above. The evidence presented here indicates that *L. oenos* takes up malate via the combined action of a $malate^-/H^+$ symport and a $malate^-$ uniport and that the energy gain from such a mechanism would account for the improved growth rate and biomass yield obtained when both glucose and malate are used as energy substrates. Resolution of how many carriers there are and what translocation they catalyze ultimately requires confirmation in reconstituted systems known to possess only one carrier at a time. The apparent dependence of the malate uniport on malate concentration rather than the concentration gradient is unusual and merits further attention.

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