

## Chemiosmotic Energy from Malolactic Fermentation

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**By using the luciferase-luciferin ATP assay and whole cells of *Leuconostoc oenos*, we have demonstrated that malolactic fermentation does yield ATP. This energy-yielding mechanism did not occur in a cell extract and was inhibited in the presence of dicyclohexylcarbodiimide or an ionophore such as monensin. A lactate:proton efflux mechanism for this proposed pathway is presented.**

During the growth of lactic acid bacteria in wine, L-malic acid is converted to L-lactic acid and CO<sub>2</sub> (15). This reaction is commonly referred to as malolactic fermentation (MLF). Species of *Leuconostoc*, *Lactobacillus*, and *Pediococcus* can carry out MLF, but *Leuconostoc oenos* is recognized as the bacterium that is most tolerant to the low pH and the alcohol content of wine (2; T. Henick-Kling, Ph.D. thesis, University of Adelaide, Adelaide, Australia, 1986). A reduction in the acidity of wine, caused by the growth of *L. oenos*, is brought about by a decarboxylating enzyme, the malolactic enzyme (EC 1.1.1.38). In the decarboxylation reaction, no pyruvate intermediate is formed and the reaction is probably catalyzed by a single enzyme which requires NAD and Mn<sup>2+</sup> for catalytic activity (13). The lack of a pyruvate intermediate prompted researchers to report that MLF serves a non-energy-yielding function (11). However, malic acid did give a slight increase in the cell yield and accelerated the rate of growth; therefore, the researchers concluded that the decarboxylation reaction stimulated the utilization of carbon sources (11). Henick-Kling (thesis) showed that the catabolism of malate by *L. oenos* and *Lactobacillus plantarum* can increase the intracellular pH and the membrane potential. The pathway may conserve some of the energy of the reaction in the form of ATP or an ion gradient. In this study we have investigated the generation of ATP by *L. oenos* upon the addition of L-malate.

*Leuconostoc oenos* Lc5b1 was obtained from the Australian Wine Research Institute, Urrbrae, South Australia. *L. oenos* Lc5b1 was grown in a modified MRS medium containing (per liter) 10 g of glucose, 10 g of fructose, 4 g of DL-malic acid, 10 g of bacteriological peptone, 8 g of casein hydrolysate, 4 g of yeast extract, 1 ml of Tween 80, 5 g of KH<sub>2</sub>PO<sub>4</sub>, 0.25 g of MgSO<sub>4</sub>, and 0.20 g of MnSO<sub>4</sub> · 1H<sub>2</sub>O. The pH of the growth medium was adjusted to 3.5 or 5.5, and the medium was sterilized at 120°C for 15 min. The cultures were inoculated at 2.0% (vol/vol) with precultures. Cells were harvested at the late exponential phase of growth by centrifugation at 8,000 × g for 15 min and washed in a tartrate-phosphate buffer, pH 3.5 or 5.5 (to match the initial pH of the growth medium), which contained 40 mM tartaric acid, 80 mM K<sub>2</sub>HPO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, and 0.5 mM MnSO<sub>4</sub>.

The amount of ATP in cells and cell extracts was measured by using a luciferase assay (8), which was modified slightly. Cells (0.5 ml at 1 to 2 mg of protein per ml) were mixed with 500 μl of 1.2 N HClO<sub>4</sub>, allowed to lyse for 2.0 min, and then centrifuged in a Beckman microcentrifuge at

13,000 × g for 2 min. Then, 20 μl of extracted sample was mixed with 2.0 ml of 0.1 M potassium phosphate, pH 7.8, and 50 μl of luciferin-luciferase assay mix at 40 mg/ml (Sigma Chemical Co., St. Louis, Mo.). Luminescence was assayed with a Tracor Delta 300 scintillation counter (Tracor Analytical, Elk Grove, Ill.). ATP concentrations during malate catabolism were assayed as above except that the cells were incubated in tartrate-phosphate buffer plus 20 mM DL-malate at pH 3.5 or 5.5 for 2.0 min prior to extraction. Cell extracts were prepared by passing a 20-ml washed-cell suspension, at 1 to 2 mg of protein per ml of cells in tartrate-phosphate buffer, twice through an Aminco model FA-073 French pressure cell at 8.274 MPa (SLM Instruments, Urbana, Ill.). Whole cells were treated with *N,N'*-dicyclohexylcarbodiimide (DCCD; Sigma Chemical Co.) at 10<sup>-4</sup> M and monensin (Sigma Chemical Co.) at 5 × 10<sup>-6</sup> M. Protein concentration determinations were done by the method of Bradford (1).

Incubation of cells in tartrate-phosphate buffer of pH 3.5 or 5.5 caused a decrease in ATP content (Fig. 1A and B). ATP concentrations decreased to half the original concentration with a half-time of approximately 20 min. The ATP concentration reached a level of less than 1.0 nmol of ATP per mg of protein within 120 min.

In order to test whether malate is used as an energy

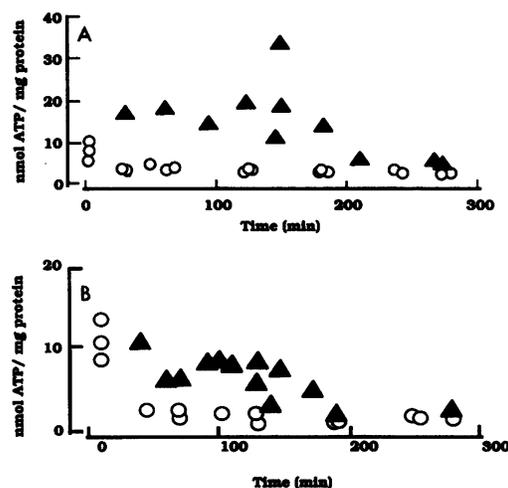


FIG. 1. Effect of malate on decay of the ATP pool in cells of *L. oenos* in tartrate-phosphate buffer. Cells of *L. oenos* were incubated in tartrate-phosphate buffer of pH 3.5 (A) and 5.5 (B). Circles represent ATP content during starvation decay; triangles represent malate-dependent ATP production during starvation.

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TABLE 1. Effect of various cell treatments on malate-dependent ATP production

Cells and treatment	ATP production (nmol/mg of protein) in tartrate-phosphate buffer at:	
	pH 3.5	pH 5.5
Whole cells, no malate	0.0446	0.0251
Whole cells, 20 mmol L-malate	10.000	7.940
Cell extract, <sup>a</sup> 20 mmol L-malate	0.0561	0.0547
Whole cells, 20 mmol L-malate, $5 \times 10^{-6}$ M monensin	0.00346	0.00576
Whole cells, 20 mmol L-malate, $10^{-4}$ M DCCD	0.126	0.141

<sup>a</sup> Cell extract was suspended in buffer of pH 6.0 to maintain malolactic enzyme activity.

source, 20 mM DL-malate at pH 3.5 and 5.5 was added to cells starved for various times in buffer of pH 3.5 and 5.5, respectively. Figure 1A compares the ATP decay in cells suspended in tartrate-phosphate buffer of pH 3.5 with the ATP content of starved cells which catabolized 20 mM DL-malate in tartrate-phosphate buffer of the same pH for 2.0 min prior to the ATP assay. The increase in ATP concentration during malate catabolism was generally an order of magnitude greater than starvation levels. The initial concentration of ATP before the addition of malate was less than 1.0 nmol of ATP per mg of protein; after the addition of malate, a maximum response of 30.77 nmol of ATP per mg of protein was measured. The quantity of the ATP response varied depending on the time elapsed between harvesting and measuring of the ATP content (starvation time). The largest response was generated for freshly harvested cells which were assayed after harvest, wash, and resuspension procedures at a constant time of 15 min from start of harvest to time of the ATP assay. After prolonged starvation, progressively smaller ATP concentrations were measured upon the addition of DL-malate. This suggests that the amount of ATP produced during L-malate catabolism is directly related to metabolic activity. Cells of *L. oenos* grown in medium of pH 5.5 showed a similar type of response (Fig. 1B).

Cell extract containing active malolactic enzyme in buffer of pH 6.0 produced no malate-dependent ATP response; however, whole cells of *L. oenos* suspended in tartrate-

phosphate buffer with malate showed a response of 10.0 and 7.94 nmol of ATP per mg of protein, respectively, at pH 3.5 and 5.5 (Table 1). Monensin, an ionophore, inhibited ATP synthesis in whole cells during malate catabolism at either pH 3.5 or pH 5.5 (Table 1). The use of an ATPase inhibitor, DCCD, gave similar results (Table 1).

There have been several attempts to study the energetics of the malolactic decarboxylation reaction (4, 10, 11, 13). These studies used cell extracts and purified enzyme. The results of these experiments suggest that there is no substrate-level phosphorylation associated with MLF. This was confirmed during this investigation by our measurements with cell extract.

The measured increase in ATP concentration in cells of *L. oenos* catabolizing malate shows that whole, intact cells generate the ATP response observed in this study. Apparently, an electrochemical or proton gradient generated across the cell membrane is necessary for ATP production. The inhibition of ATP synthesis by monensin, which dissipates a proton gradient, indirectly shows that this gradient is necessary for the production of ATP in cells of *L. oenos* catabolizing malate. Since the intact cell is a necessary component of this energy generation scheme, we propose that a chemiosmotic theory for the production of ATP from malate catabolism can be advanced.

The chemiosmotic theory as proposed by Mitchell (6, 7) does not provide for end product efflux as a means of creating a proton gradient. Michels et al. (5) proposed that the generation of lactate from glucose catabolism is capable of developing a proton gradient as lactate plus  $nH^+$  is excreted. MLF is a decarboxylation reaction of L-malate to L-lactate and  $CO_2$ . The excreted end products of the malolactic reaction may be capable of generating a proton gradient. We propose that the ability of a cell to expel lactate and  $CO_2$  in symport with  $n$  protons ( $n$  being between 1 and 2  $H^+$ ) can theoretically generate the proton gradient during the malolactic reaction. The cell membrane is, of course, an integral part of this theory, as is the ATPase. Measurements of the membrane potential in cells of *L. oenos* have shown that the membrane potential during the catabolism of malate has a value of 258 mV (Henick-Kling, thesis). The measured potential is sufficient to drive the generation of ATP via the ATPase. It was observed in this study that when the ATPase is inhibited with DCCD, or when an ionophore is used, the generation of ATP during malate catabolism ceases. A

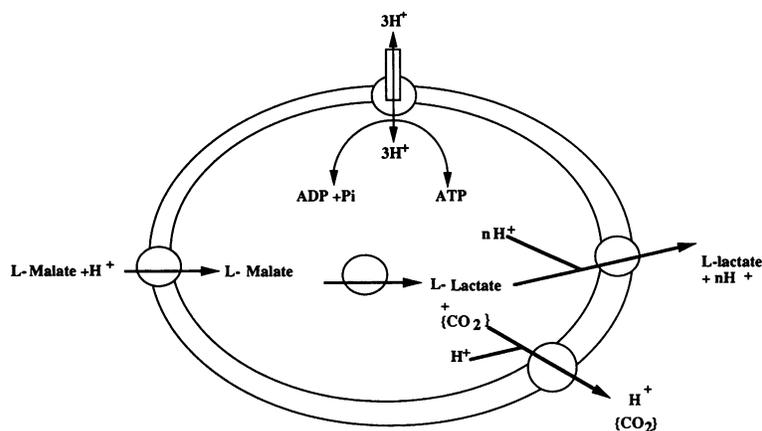


FIG. 2. Hypothetical ATP-generating chemiosmotic mechanism for the malolactic enzyme system. The malolactic enzyme, shown as a circle in the center of the figure, can decarboxylate malate in a single non-energy-yielding step. The electrogenic transport of lactate and/or carbon dioxide (brackets enclosing  $CO_2$  indicate that the mechanism is not defined) would be responsible for any energy produced.

hypothetical mechanism for the generation of ATP using end product efflux and the malolactic enzyme is presented in Fig. 2. The malolactic enzyme may be directly or indirectly (peripheral or soluble) associated with the lactate:H<sup>+</sup> symport enzyme. The number of protons excreted as a result of malate catabolism is not yet known; however, previous reports have put this number between 1 and 2 protons for end product efflux for related organisms (3, 9, 14).

A second possibility is that the increase in intracellular pH generated during malolactic decarboxylation may make other cellular reactions more favorable or reduce the need to use ATP to maintain pH homeostasis. Thus, malate catabolism might indirectly contribute to the conservation of available ATP but not be directly involved in its synthesis. However, the quantity of ATP produced during malate catabolism is large enough to support the theory of direct chemiosmotic ATP synthesis.

Further work will survey other lactic acid bacteria containing the malolactic enzyme system for energy production and attempt to define the energy-yielding mechanism of the malolactic decarboxylating reaction.

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#### LITERATURE CITED

1. Bradford, M. M. 1976. A rapid and sensitive method for the determination of microgram quantities proteins utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248-254.
2. Henick-Kling, T., T. H. Lee, and D. J. D. Nicholas. 1986. Inhibition of bacterial growth and malolactic fermentation in wine by bacteriophage. *J. Appl. Bacteriol.* **61**:287-293.
3. Konings, W. N., and R. Otto. 1983. Energy transduction and solute transport in streptococci. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **49**:247-257.
4. Kunkee, R. E. 1975. A second enzymatic activity for decomposition of malic acid by malo-lactic bacteria, p. 29-42. In J. G. Carr, C. V. Cutting, and G. C. Whiting (ed.), *Lactic acid bacteria in beverages and food*. Academic Press, Inc. (London), Ltd., London.
5. Michels, P. A. M., J. P. J. Michels, J. Boonstra, and W. N. Konings. 1979. Generation of an electrochemical proton gradient in bacteria by the excretion of metabolic end products. *FEMS Microbiol. Lett.* **5**:357-364.
6. Mitchell, P. 1966. Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev. Proc. Camb. Phil. Soc.* **41**:445-502.
7. Mitchell, P. 1972. Chemiosmotic coupling in energy transduction: a logical development of biochemical knowledge. *J. Bioenerg.* **3**:5-24.
8. Nguyen, V. T., M. Morange, and O. Bensaude. 1988. Firefly luciferase luminescence assays using scintillation counters for quantitation in transfected mammalian cells. *Anal. Biochem.* **171**:404-408.
9. Otto, R., S. M. Sonnenberg, H. Veldkamp, and W. N. Konings. 1980. Generation of an electrochemical proton gradient in *Streptococcus cremoris* by lactate efflux. *Proc. Natl. Acad. Sci. USA* **77**:5502-5506.
10. Pilone, G. J., and R. E. Kunkee. 1970. Carbonic acid from decarboxylation by "malic" enzyme in lactic acid bacteria. *J. Bacteriol.* **103**:404-409.
11. Pilone, G. J., and R. E. Kunkee. 1972. Characterization and energetics of *Leuconostoc oenos*. *Am. J. Enol. Viticult.* **23**: 61-70.
12. Rottenberg, H. 1979. The measurement of membrane potential and  $\Delta\text{pH}$  in cells, organelles and vesicles. *Methods Enzymol.* **55**:547-569.
13. Schütz, M., and F. Radler. 1973. Das "Malatenzym" von *Lactobacillus plantarum* und *Leuconostoc mesenteroides*. *Arch. Mikrobiol.* **91**:183-202.
14. Ten Brink, B., R. Otto, U. P. Hansen, and W. N. Konings. 1985. Energy recycling by lactate efflux in growing and nongrowing cells of *Streptococcus cremoris*. *J. Bacteriol.* **162**:383-390.
15. Wibowo, D., R. Eschenbruch, C. R. Davis, G. H. Fleet, and T. H. Lee. 1985. Occurrence and growth of lactic acid bacteria in wine: a review. *Am. J. Enol. Viticult.* **36**:302-313.