Energy production from L-malic acid degradation and protection against acidic external pH in *Lactobacillus plantarum* CECT 220

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Malate degradation by *Lactobacillus plantarum* CECT 220 provides energy which enables this organism to remain viable for longer at low environmental pH values. Energy production was not coupled to H⁺-ATPase activity. This protective mechanism against acidic external pH is complemented by another system of ΔpH maintenance. The H⁺-ATPase did not seem to be involved in this system of pH maintenance as the system was not sensitive to *N,N*-dicyclohexylcarbodiimide (DCCD) and was functional at very low pH values where ATPase activity was severely inhibited.

Introduction

Lactic acid bacteria (LAB) are obligate fermentative micro-organisms. As they produce large amounts of acid, tolerance to acidic environments is a valuable asset for their survival. This tolerance is particularly advantageous in vegetable fermentations and winemaking where LAB play an important role (Stamer, 1979).

The adaptation of these micro-organisms to low pH implies the development of protective mechanisms such as the arginine deiminase system proposed by Marquis *et al.* (1987). A similar role has been proposed for malo-lactic fermentation (MLF) (Kunkee, 1967; Caspritz & Radler, 1983). MLF is the decarboxylation of L-malic acid to L-lactic acid by the malo-lactic enzyme (MLase), which is only found in LAB. This reaction does not seem to provide energy to the cells (Kunkee, 1967; Daeschel, 1988) but appears to be beneficial, because their growth rates are increased when grown in a medium with L-malic acid and a carbon source (Pilone & Kunkee, 1976). Recently, Cox & Henick-Kling (1989) proposed a chemiosmotic hypothesis for MLF in *Leuconostoc oenos* where energy is produced by the efflux of L-lactate from L-malate degradation. It has also been shown that other LAB, including *Lactobacillus plantarum*, can obtain energy from L-malic acid degradation (Cox & Henick-Kling, 1990).

L. plantarum is one of the most widespread LAB. It has been isolated from dairy products, meat and meat products, silage, sugar processing, fruit juices, and man and other animals. Furthermore, it is commonly used in vegetable fermentations and winemaking as a starter culture (Prahl *et al.*, 1988). The MLase of *L. plantarum* has been purified (Schütz & Radler, 1973). As no malic enzyme activity has been detected in *L. plantarum* (Caspritz & Radler, 1983; Battermann & Radler, 1991), pyruvic acid production from L-malic acid, which could be diverted to energy generation, is not expected.

The aim of this work was to determine the role of MLF at low pH values and to investigate whether malo-lactic degradation could provide energy for *L. plantarum*. The results show several novel aspects of this process.

Methods

Organisms and growth media. *L. plantarum* CECT 220 was from the Colección Española de Cultivos Tipo (CECT, Universidad de Valencia, Spain). Cultures were maintained by weekly transfers in de Man, Rogosa, and Sharpe medium (MRS) (Oxoid) (de Man *et al.*, 1960) supplemented with 0.3% agar. For longer conservation the cultures were lyophilized. *L. plantarum* CECT 220 was grown routinely in MRS broth at 30 °C in static culture. MRS agar plates were prepared by supplementing broth with 2% (w/v) agar.

Determination of minimum pH values for glucose metabolism and growth. Determination of minimum pH value for glucose metabolism was carried out as described by Casiano-Colón & Marquis (1988). Cells were harvested in early stationary phase, centrifuged, washed twice in 20 mM-potassium phosphate buffer, pH 5.5, containing 1 mM-MgCl₂, and suspended (2 mg dry weight ml⁻¹) in the same buffer. Glucose was added to a final concentration of 20 g l⁻¹ to give an excess. Since LAB

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Abbreviations: LAB, lactic acid bacteria; MLF, malo-lactic fermentation; MLase, malo-lactic enzyme; PCA, perchloric acid.

are obligate fermenters, the metabolism of glucose leads to a decrease of the medium pH. External pH values were measured with an Orion (SA 520) pH meter. When pH readings were stable, the pH of the medium was increased to the initial pH value with sterile 1 M-KOH and pH measurements continued. A further decrease in pH indicated that the minimum pH for glucose metabolism had been reached. The minimum pH value for growth was obtained by culturing *L. plantarum* CECT 220 in MRS broth at 30 °C at different initial pH values. Growth was monitored as OD₆₀₀ with a Beckman DU-7 spectrophotometer.

Culture viability with and without L-malic acid at different pH values, and determination of minimum pH value for degradation of L-malic acid. Cells were harvested as described above, washed twice with 20 mM-potassium phosphate buffer containing 1 mM-MgCl₂ at the desired pH value and suspended in the same buffer with or without 5 g l⁻¹ L-malic acid to obtain dense suspensions containing about 10⁹ c.f.u. ml⁻¹. Incubation was carried out at 30 °C. The pH values used were 3.0 and 5.5. Samples were taken periodically and plated on MRS agar at pH 6.0. Plates were incubated at 30 °C for 48 h before counting.

Quantification of organic acids concentration. Degradation of L-malate was followed by the UV method for determination of residual L-malic acid (Boehringer Mannheim, Barcelona). L-Lactic acid, D-lactic acid, and acetic acid were also measured similarly. Changes in external pH values were monitored simultaneously.

Measurements of ΔpH. ΔpH (=pH_i-pH_o, where pH_i is the internal pH and pH_o is the external pH) was measured from the distribution of [¹⁴C]salicylic acid (10 μM, 0.05 nCi μl⁻¹, 1.85 Bq μl⁻¹) as described (Kobayashi *et al.*, 1982) with modifications. To calculate pH_i, the data were corrected since the pH_o was lower than the pK_a+1 of the radiolabelled marker (Rottenberg, 1979). This means that a significant part of the marker is not dissociated, so pH_i was calculated from:

$$\text{pH}_i = \log \left[\frac{([A]_i/[A]_o)(10^{\text{pK}_a} + 10^{\text{pH}_o}) - 10^{\text{pK}_a}}{1} \right]$$

where [A]_i and [A]_o are the internal and external concentration of radiolabelled marker (dissociated and not dissociated) respectively.

Cells were suspended in 20 mM-potassium phosphate buffer (pH 3.0 or 5.5) containing 1 mM-MgCl₂. [¹⁴C]salicylic acid was added and the suspension was incubated at 30 °C for at least 10 min. Samples (50 μl) were analysed by scintillation spectroscopy (Packard 3255) in vials containing Bray's solution. The remaining part of the suspension was filtered through a glass microfibre filter (Whatman GF/F) and the radioactivity on the filter determined. Replacement of [¹⁴C]salicylic acid by [¹⁴C]benzoic acid did not affect the results obtained (data not shown). As a positive control, 1% (w/v) glucose was added to the cell suspension, and incubated for 10 min. Small ΔpH were corrected by subtracting the ΔpH values obtained in the presence of gramicidin D (4 μg ml⁻¹). *N,N'*-Dicyclohexylcarbodiimide (DCCD) was added (final concentration, 0.2 mM) to inhibit ATPase activity. Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was used at a final concentration of 20 μM. Ionophores and uncouplers used were allowed to act for 20 min before measurements of ΔpH. The internal volume of 3.2 μl (mg protein)⁻¹ of *L. plantarum* CECT 220 was determined as described (Rottenberg, 1979).

Everted membrane vesicles. Membrane vesicles were obtained as described (Russell *et al.*, 1988) except that the vesicles were resuspended in 2 ml 50 mM-sodium maleate buffer, pH 7.0, containing 10 mM-MgSO₄ (final protein concentration 9.4 mg protein ml⁻¹). Everted membrane vesicles were obtained by passing the final suspension through a French pressure cell at 703 kg cm⁻² (Aminco) and stored at -80 °C until use.

Assay of ATPase activity and measurements of intracellular ATP. Measurements of ATPase activity were performed by determining P_i released from ATP (Kobayashi & Anraku, 1972). The procedure was

carried out using everted membrane vesicles as a source of ATPase activity.

ATP concentrations in cell extracts were measured using the luciferin-luciferase method (Lundin & Thore, 1975) with a 1234-102 ATP Monitoring Kit (LKB Wallac, Barcelona) and a LKB Wallac 1250 luminometer. Cells were grown in MRS broth at 30 °C until early stationary phase, harvested by centrifugation (15 300 g, 20 min, 4 °C) and suspended in 20 mM-potassium phosphate buffer containing 1 mM-MgCl₂ (pH 3.0 or 5.5) to a concentration of 3–4 mg protein ml⁻¹. Samples (4 ml) were taken after 24 h incubation at 30 °C in the buffer, and combinations of L-malic acid (5 g l⁻¹), DCCD (0.2 mM), glucose (1% w/v), and gramicidin D (4 μg ml⁻¹) added. Cells were incubated for 10 min at 30 °C after addition of the compounds. None of the treatments altered the pH_o. One ml of treated cells was extracted as described (Strobel & Russell, 1989) with 0.5 ml cold 14% (v/v) PCA containing 9 mM-EDTA for 20 min on ice. Cell debris was removed by centrifugation (13 000 g, 5 min, 4 °C). One ml of supernatant was neutralized with 0.5 ml KOH/KHCO₃. Precipitates were removed by centrifugation (13 000 g, 5 min, 4 °C), and the supernatants stored at -20 °C until use.

Protein determination. The amount of protein was determined by the method of Lowry using bovine serum albumin as standard.

All measurements were performed at least in duplicate. The results given are the average.

Results

Growth, cell viability, and L-malate degradation by L. plantarum

Non-growing cells of *L. plantarum* CECT 220 degraded glucose until the external pH reached 2.7, whereas L-malic acid could be degraded down to external pH 2.4. In MRS broth, cell growth stopped at pH 3.1, but glucose continued to be degraded until pH 2.95 was reached. The minimum pH values for glucose degradation obtained with non-growing and growing cells are not similar, because acid tolerance changes depending on the environmental conditions (Casiano-Colón & Marquis, 1988).

Cells remained viable longer in the presence of L-malic acid in buffers at either pH 3.0 or 5.5 (Fig. 1*a*). L-Malic acid degradation activity was observed at pH 3.0. At an initial pH of 5.5, however, L-malic acid was not completely depleted; approximately 1 g l⁻¹ remained after 220 h of incubation (Fig. 1*b*). The cell viability at both pH 3.0 and 5.5 remained almost unchanged until malic acid degradation stopped, after which viability decreased, but not as rapidly as in the absence of malate (Fig. 1*a,b*). Degradation of L-malic acid was accompanied by an increase in external pH, as expected. The external pH values rose from 3.0 to 3.4, and from 5.5 to 7.5 after 120 h of incubation. Despite the more effective metabolism of L-malate at pH 3.0, a greater increase of external pH was detected at pH 5.5. Although a slight increase of external pH at initial pH 3.0 occurred

Fig. 1. Viability (a) and degradation of L-malic acid (b) as a function of time at initial external pH values 3.0 and 5.5, by *L. plantarum* CECT 220 cells suspended in 20 mM potassium phosphate buffer containing 1 mM-MgCl₂. ■, L-malic acid added, pH 5.5; ◆, no L-malic acid added, pH 5.5; □, L-malic acid added, pH 3.0; ◇, no L-malic acid added, pH 3.0; ▲, pH 5.5; △, pH 3.0.

(only 0.4 units), the cells remained viable longer in presence of L-malic acid.

The main products of L-malic acid degradation by *L. plantarum* CECT 220 at pH 5.5 were L- and D-lactic acids (Fig. 2). Minor amounts of acetic acid were also produced (Fig. 2). Similar results were obtained when cells were incubated at pH 3.0 (data not shown). CO₂ production was not measured in these experiments. It is generally accepted that MLF yields only L-lactic acid and CO₂ but our results do not bear this out. This discrepancy is discussed later.

Generation of a ΔpH in non-growing cells at pH 3.0 and 5.5 with or without L-malate

Starvation of cells (incubation without L-malic acid) resulted in a very rapid decrease in the ΔpH and

viability. High ΔpH s were generated at pH 3.0 when cells were incubated with L-malic acid (Table 2), whereas a low ΔpH was detected at pH 5.5. This could be due to the lower L-malic acid degradation rate at pH 5.5 (Fig. 1b). Cells were incubated in buffer at pH 3.0 in the presence or absence of 5 g of L-malic acid l⁻¹ for 6 h. Gramicidin D and CCCP dissipated the ΔpH (Table 2), which suggested that circulation of protons takes place upon addition of L-malic acid. Nevertheless, since the dissipation by ionophores was not complete, a Donnan potential may contribute partially to generation of ΔpH at low pH. When cells were treated with DCCD at pH 5.5, the ΔpH was completely dissipated, both in the presence of malate (in which case the external pH reached a value of 5.9 due to malate degradation) and glucose (Table 1). These results indicated that H⁺-ATPase was involved in maintaining the ΔpH at pH 5.5. In contrast, cells treated with DCCD at pH 3.0 were able

Fig. 2. Degradation of L-malic acid and production of L-lactic acid, D-lactic acid, and acetic acid by non-growing cells suspended in potassium phosphate buffer at an external pH of 5.5. ■, L-malic acid; ♦, L-lactic acid; □, D-lactic acid; ◇, Acetic acid.

Table 1. Intracellular ATP of cells suspended in 20 mM-potassium phosphate buffer at external pH values of 3.0 or 5.5 after 24 h incubation at 30 °C

Treatment	pmol ATP (mg protein) ⁻¹	
	pH _o 3.0	pH _o 5.5
Untreated cells	46	43
L-Malic acid	210	200
DCCD + L-malic acid	260	220
Glucose	440	170
DCCD + glucose	500	250
L-Malic acid + gramicidin D	9	0

to maintain the Δ pH in the presence of glucose and L-malate (Table 2). In this context it is possible that DCCD was somehow unable to inhibit ATPase at pH 3.0. However, ATPase activity in everted membrane vesicles was sensitive to DCCD at both pH 3.0 and 5.5 (data not shown).

ATP was produced upon addition of L-malic acid at both pH 3.0 and 5.5 (Table 1). ATP was produced from malate degradation even if cells were treated with DCCD. This suggests that L-malate degradation provides energy in a way that does not involve ATPase activity.

Table 2. Measurement of Δ pH in *L. plantarum* CECT 220 cells incubated in 20 mM-potassium phosphate buffer at initial external pH values of 3.0 or 5.5

Incubation time (h)	Treatment	pH _o 3.0		pH _o 5.5	
		pH _o	Δ pH	pH _o	Δ pH
0	None	3.0	1.6	5.5	0.4
0	L-Malic acid	3.0	2.2	5.5	0.5
0	Glucose	3.0	2.4	5.5	1.8
6	None	3.0	1.2	5.1	No Δ pH
6	L-Malic acid	3.1	1.8	5.9	No Δ pH
6	L-Malic acid + DCCD	3.1	1.7	5.9	No Δ pH
6	L-Malic acid + CCCP	3.1	1.1	ND	ND
6	L-Malic acid + gramicidin D	3.1	0.7	ND	ND
6	Glucose	3.0	2.4	5.1	1.7
6	Glucose + DCCD	3.0	2.4	5.1	No Δ pH

ND, not determined.

Discussion

The MLase activity of whole cells suspended in buffer was higher at pH 3.0 than at pH 5.5 (Fig. 1*b*); similar to the results of Henick-Kling (1986). However, the optimum pH for MLase activity of the purified enzyme is around 5.5–6.0, (Kandler *et al.*, 1973; Strasser de Saad *et al.*, 1984). Possible explanations are that malic acid permease has a lower optimum pH than malo-lactic enzyme, or that the regulatory system of malo-lactic process is pH-dependent. The results showed that the minimum pH for MLF (pH 2.4) was lower than that for glycolysis (pH 2.7), and growth (pH 3.1). Moreover, cells remained viable longer in buffer with L-malic acid (Fig. 1*a*), and MLF activity was higher at pH 3.0 than at 5.5. These results indicate that MLF in *L. plantarum* CECT 220 is a protective system in acidic environments.

The proposed mechanism for MLF includes neither the formation of ATP nor the reduction of NAD (Kunkee, 1967). Kandler *et al.* (1973) suggested that the stimulatory effect on the growth of LAB by the MLF was due exclusively to the increase of the environmental pH. This has also been suggested with the arginine deiminase system (Marquis *et al.*, 1987; Casiano-Colón & Marquis, 1988), but arginine degradation is accompanied by a high increase in external pH as NH_3 is produced. We observed that incubation of cells in buffer at pH 3.0 with L-malic acid only produced a slight increase of the external pH, but this increase is insufficient to explain the higher viability obtained in the presence of L-malic acid at pH 3.0, since cells incubated at pH 5.5 in buffer with no L-malic acid died faster than those incubated at pH 3.0 with L-malic acid (Fig. 2*a*). Conversely, our results show that *L. plantarum* CECT 220 can obtain energy from malic acid degradation even at low pH values. Significant levels of intracellular ATP were also detected upon addition of L-malic acid to cells starved for 24 h (Table 1), which means that ATP production was not due to the degradation of storage products.

Cox & Henick-Kling (1989) proposed that the L-lactic acid produced from the degradation of L-malic acid could be extruded in symport with protons to generate a membrane potential that would enable the membrane-bound H^+ -ATPase to produce ATP. This proposal was based on the observation that an electrochemical proton gradient is generated by lactate efflux (Otto *et al.*, 1980). However, ATP production in *L. plantarum* CECT 220 can not be explained by a chemiosmotic mechanism as proposed by Cox & Henick-Kling (1989), since ATP production was not affected by DCCD in cells incubated in buffer at pH 5.5 (Table 1), where the ATPase was effectively inhibited.

To clarify the origin of this ATP production, the products from L-malate degradation by cells suspended

in buffer were analysed. According to the mechanism generally accepted for MLF, no D-lactic acid production would be expected from malate degradation by MLase (Kunkee, 1967). However, we detected high levels of D-lactate (Fig. 2), which could be explained if *L. plantarum* CECT 220 possessed malic enzyme. Nevertheless, no malic enzyme activity has been detected in this species (Battermann & Radler, 1991). The only LAB with malic enzyme are *Enterococcus faecalis* and *L. casei*, and *L. casei* is the only one in which both enzymes have been detected (Battermann & Radler, 1991). Battermann & Radler (1991) reported that in *L. casei* malic enzyme is unstable at pH values lower than 6.0 whereas the malo-lactic enzyme is stable at pH 4.0. In this study the highest internal pH measured was 5.2, when the external pH was adjusted to 3.0, in the presence of L-malic acid. Thus, it seems unlikely that malic enzyme could account for D-lactic acid production from L-malic acid under these conditions. *L. plantarum* CECT 220 could possess racemases to convert part of the L-lactic acid produced from L-malic acid to D-lactic acid. These enzymes have been found in *L. plantarum* and *L. brevis* (Radler *et al.*, 1970). ATP production could be coupled to the formation of acetic acid which could be derived from lactic acid. In *L. plantarum*, lactate can be further degraded to pyruvic acid and acetic acid (Murphy *et al.*, 1985; Lindgren *et al.*, 1990). The present study cannot exclude either of these proposals.

L. plantarum maintains a ΔpH with malic acid at low pH. The generation of ΔpH is via the membrane-bound H^+ -ATPase in most LAB (Kobayashi *et al.*, 1982; Kashket, 1987). When cells were treated with DCCD at pH 5.5, to inhibit H^+ -ATPase, the ΔpH in the presence of either glucose or malic acid was dissipated as expected. This implied that H^+ -ATPase is involved in ΔpH maintenance at pH 5.5. However, DCCD did not dissipate the ΔpH at pH 3.0 when cells were incubated with L-malic acid or glucose (Table 2). ATPase activity was strongly inhibited by pH and the residual activity was inhibited by DCCD (data not shown). MLF increased the internal pH because one H^+ is consumed per molecule of L-malic acid degraded; thus, ΔpH generation by this process would not be sensitive to DCCD. However, the generation of a ΔpH by glucose at pH 3.0 does not produce less acidic products and the ΔpH generation was not affected by DCCD. These results cannot be completely explained by the formation of a Donnan potential, although a Donnan potential probably accounted for ΔpH , as gramicidin D and CCCP did not dissipate completely the ΔpH (Table 2).

Cox & Henick-Kling (1989) hypothesized that the ATPase was responsible for the ΔpH generation. However, Maloney (1990) postulated and Poolman *et al.* (1991) recently confirmed that a malate²⁻/lactate¹⁻

antiport exchange would generate a membrane potential, while the decarboxylation reaction established the ΔpH . This system would be insensitive to DCCD as ATPase is not involved in the generation of the ΔpH . This cannot explain the results obtained with glucose and DCCD at pH 3.0. Nevertheless, LAB can divert the pyruvate produced from the homolactic fermentation of hexoses to the production of other compounds under stress conditions (e.g. low pH) (Rhee & Pack, 1980). Recently, Tsau *et al.* (1992) reported that *L. plantarum* was able to accumulate pyruvate and, at low environmental pH values, shift to the production of acetoin (a neutral compound). In this way, a ΔpH could be generated independently from ATPase activity.

In summary, MLF is a protective system against cell death at acidic environmental pH values. This system functions by the formation of a ΔpH , generating ATP. The ΔpH is probably established by a malate²⁻/lactate¹⁻ antiport exchange, with ATP production linked to the degradation of lactate to acetate.

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