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Conversion of Pyruvate to Acetoin Helps To Maintain pH Homeostasis in *Lactobacillus plantarum*†

JYA-LI TSAU,[1] ARTHUR A. GUFFANTI,[2] AND THOMAS J. MONTVILLE[1]*

Department of Food Science, New Jersey Agricultural Experiment Station, Cook College, Rutgers—the State University, New Brunswick, New Jersey 08903-0231,[1] and Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029[2]

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Pyruvate is the substrate for diacetyl and acetoin synthesis by lactobacilli. Exogenous pyruvate stimulates acetoin production when glucose is present as an energy source. In *Lactobacillus plantarum* ATCC 8014, the energy derived from glucose via glycolysis generated a constant proton motive force of about −120 mV. At a low external pH, energized cells rapidly transported and accumulated pyruvate but did not do so when they were deenergized by nigericin. When large amounts of pyruvate were transported and subsequently accumulated internally, the cotransported protons rapidly lowered the internal pH. The conversion of pyruvate to acetoin instead of acidic end products contributed to the maintenance of pH homeostasis. This is the first report showing that the conversion of pyruvate to acetoin serves as a mechanism of pH homeostasis.

Pyruvate is a key catabolic intermediate in many lactic acid bacteria and is converted into a variety of end products, such as lactic acid, acetic acid, acetaldehyde, ethanol, diacetyl, and acetoin (13, 14). The characteristic buttery and nutlike flavor of dairy products is attributed to diacetyl and acetoin (11), which are formed by two metabolic pathways that both require pyruvate as a precursor (17, 26). In homofermentative lactic acid bacteria, most of the pyruvate must be converted to lactate to regenerate NAD. Thus, little or no diacetyl and acetoin are produced unless an additional source of pyruvate, such as citrate, is present (4, 9, 15, 27). Therefore, Gunsalus (7) proposed that a high concentration of intracellular pyruvate is required for diacetyl and acetoin synthesis. Harvey and Collins (8, 10) expanded the hypothesis of Gunsalus and proposed that lactic acid bacteria produce diacetyl and acetoin to dispose of toxic amounts of pyruvate not required for biosynthesis (10).

Exogenous pyruvate enhances acetoin production in *Lactobacillus plantarum* (19, 21, 22, 27). When the organism is grown aerobically, acetoin production increases even in the absence of exogenous pyruvate (29). This occurs because NADH oxidase and acetoin dehydrogenase activities increase but NADH-independent lactate dehydrogenase activity decreases (30). In this article, we report that high concentrations of intracellular pyruvate were essential for acetoin synthesis and that the production of acetoin helped negate the drop in internal pH (pH_i) caused by pyruvate accumulation.

MATERIALS AND METHODS

Culture conditions and pyruvate utilization assay. Culture conditions and the pyruvate utilization assays were previously described (27). Metabolic inhibitors were used to study the effect of proton motive force (PMF) on pyruvate utilization. Carbonyl cyanide *m*-chlorophenylhydrazone (200 μM), *N,N'*-dicyclohexylcarbodiimide (200 μM), 2,4-dinitrophenol (200 μM), and nigericin (1 μM) were added to the cell suspension simultaneously with glucose and incubated at 25°C for 15 min. Pyruvate was then added. Pyruvate utilization and acetoin production were then determined by high-performance liquid chromatography (HPLC) (23) after 30 min of incubation.

Determination of intracellular pyruvate concentration. *L. plantarum* grown for 12 h in LCM medium (6) containing 10 mM glucose was centrifuged, washed, and resuspended in 0.4 M potassium phosphate buffer (pH 5.0, 5.5, and 6.5) containing 10 mM $MgSO_4$ to a cell density of 1 mg (cell dry weight) per ml. The cells were energized with 20 mM glucose for 15 min before pyruvate addition. At time zero and at 0.5, 3, 5, and 10 min, 1 ml of the cell suspension was withdrawn and centrifuged through silicone oil (density, 1.03). The cells and adhering water were collected in 0.15 ml of 20% perchloric acid at the bottom of the microcentrifuge tubes. The perchloric acid fraction was then mixed completely and neutralized with 3 N KOH. The pyruvate concentration in the neutralized mixture was calculated from the change in A_{340} caused by the conversion of pyruvate to lactate in the presence of excess lactate dehydrogenase and NADH (Sigma). Cells treated with 7% *n*-butanol or 1 μM nigericin for 30 min were used to determine the nonspecific binding of pyruvate, which was subtracted during the calculation of the intracellular pyruvate concentration. The conversion of [^{14}C]pyruvate to [^{14}C]acetoin was done as described above, except that [^{14}C]pyruvate was used. The amounts of pyruvate utilized and acetoin produced were determined by HPLC (23) by using a Bio-Rad Animex HPX-87H column (300 by 7.8 mm). Fractions were collected, and radioactivity was counted on a scintillation counter.

Measurement of PMF. The PMF was determined as described by Rottenberg (25) by using appropriate controls to subtract nonspecific binding. Cellular suspensions [0.3 to 0.5 mg (cell dry weight) per ml] were incubated in 0.4 M potassium phosphate buffer (pH 4.5 to 7.0) containing 10 mM $MgSO_4$. Glucose (20 mM) was added to reenergize the cells for 15 min. [^{14}C]salicylic acid (5 μM) or [^{3}H]tetraphenylphosphonium (5 μM) were added, and the mixture was incubated for 10 min at room temperature. Cell suspensions were then transferred to microcentrifuge tubes and separated by centrifugation through silicone oil.

* Corresponding author.

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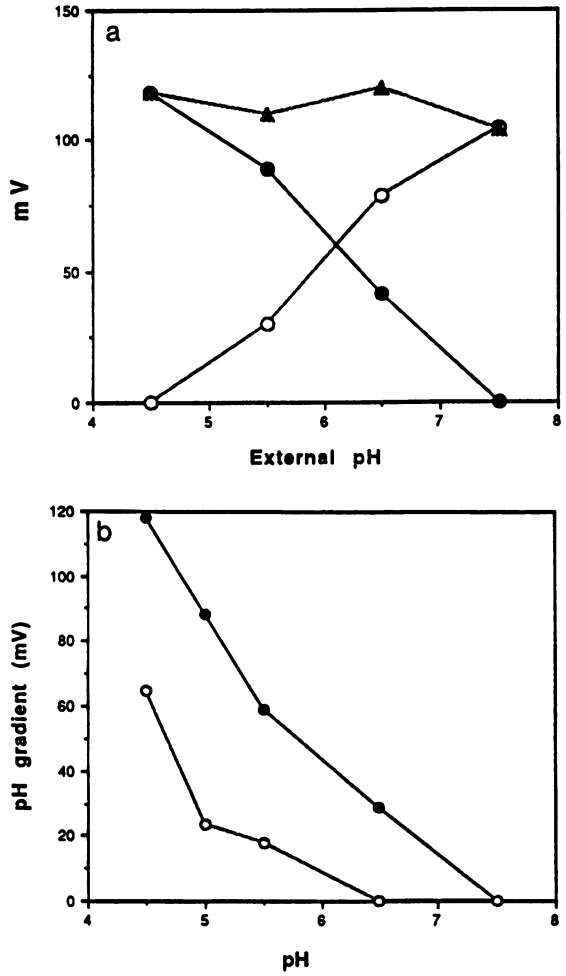

FIG. 1. (a) PMF in *L. plantarum* cells undergoing glycolysis. Symbols: ●, ΔpH; ○, ΔΨ; ▲, PMF. (b) Effect of energy source on the magnitude of ΔpH in *L. plantarum*. Cells resuspended in 0.4 M potassium phosphate buffer (pH 4.5 to 7.5) were first energized with 15 mM glucose for 15 min before [^{3}H]tetraphenylphosphonium and [^{14}C]salicylic acid were added. Symbols: ●, energized with glucose; ○, not energized.

The intracellular volume was calculated by using ^3H_2O to determine the total aqueous space and then subtracting the space occupied by [^{14}C]inulin. The calculated intracellular volume was 1.67 μl/mg (cell dry weight).

Materials. Radioactively labeled [3-^{14}C]pyruvate (16.3 mCi/mmol), [7-^{14}C]salicylic acid (58.2 mCi/mmol), and [carboxy-^{14}C]inulin (2.2 mCi/g) were purchased from New England Nuclear, Boston, Mass. [phenyl-^{3}H]tetraphenylphosphonium (62 mCi/mg) was purchased from Amersham, Arlington Heights, Ill. *N,N'*-Dicyclohexylcarbodiimide, nigericin, 2,4-dinitrophenol, and carbonyl cyanide *m*-chlorophenylhydrazone were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

PMF in *L. plantarum*. The conversion efficiency of pyruvate to acetoin increases as the external pH (pH_e) decreases (19, 27). Evidence for the active transport of pyruvate by a PMF-dependent symport includes the 135-fold accumulation

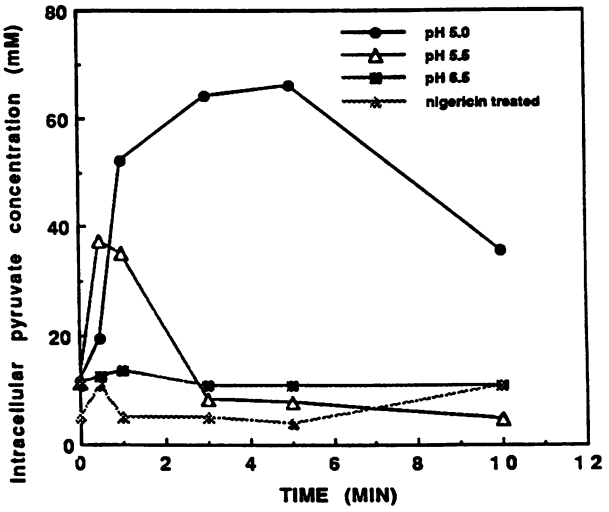

FIG. 2. Transport and accumulation of pyruvate in *L. plantarum* whole cells. Cells resuspended in 0.4 M potassium phosphate buffer (pH 5.0, 5.5, and 6.5) were energized with 15 mM glucose for 15 min before pyruvate was added at a final concentration of 5 mM. Nigericin was added at a final concentration of 1 μM.

of pyruvate against a gradient in *L. plantarum* membrane vesicles, carrier-dependent saturation kinetics having an apparent K_m of 35 μM, and both transport rate and accumulation that increase with ΔpH (i.e., $pH_i - pH_e$) but not electrical potential (ΔΨ) (28). Therefore, the effects of the pH_e and energy source on the PMF were determined (Fig. 1a and b). In the presence of glucose as an energy source (Fig. 1a), the pH_i decreased from 7.0 to 6.3 as the pH_e was decreased from 7.0 to 4.5. Thus, the ΔpH increased with decreasing pH_e. The ΔΨ decreased correspondingly so that the total PMF (negative and alkaline inside) was maintained constant around −120 mV. In the absence of glucose (Fig. 1b), the pH_i was lower, decreasing the ΔpH about 1 pH unit below the ΔpH maintained in the presence of glucose.

Accumulation of pyruvate in the resting cells. To demonstrate that high concentrations of intracellular pyruvate were required for acetoin synthesis and to confirm intracellular pyruvate accumulation against a concentration gradient, the intracellular pyruvate concentration was measured (Fig. 2). The basal (time zero) intracellular pyruvate concentration was high (about 10 mM) but similar to that reported by Mizushima and Kitahara (20). Pyruvate accumulation was immediate and rapid after its addition to the cell suspensions at pH_e 5.0 and 5.5. The intracellular pyruvate concentration decreased after 5 min, suggesting depletion of the pyruvate pool via its conversion to acetoin (27). Pyruvate accumulated about 7-fold over its external concentration at pH 5.0, 4-fold at pH 5.5, and 1.5-fold at pH 6.5. Nigericin-treated cells did not accumulate pyruvate.

Influence of pyruvate and proton influx on pH_i. Intracellular pH homeostasis in *L. plantarum* might be disturbed when pyruvate is rapidly transported, since a proton(s) is cotransported (28). Therefore, the pH_i was measured after pyruvate addition (Fig. 3a). In the presence of 20 mM external pyruvate, the pH_i dropped from 6.3 to 5.5 within 10 min. When the pyruvate pool was high, the conversion of pyruvate to acetoin and lactate occurred; the pH_i increased gradually (Fig. 3b). The presence of [^{14}C]lactate indicates that some of the transported pyruvate entered the intracel-

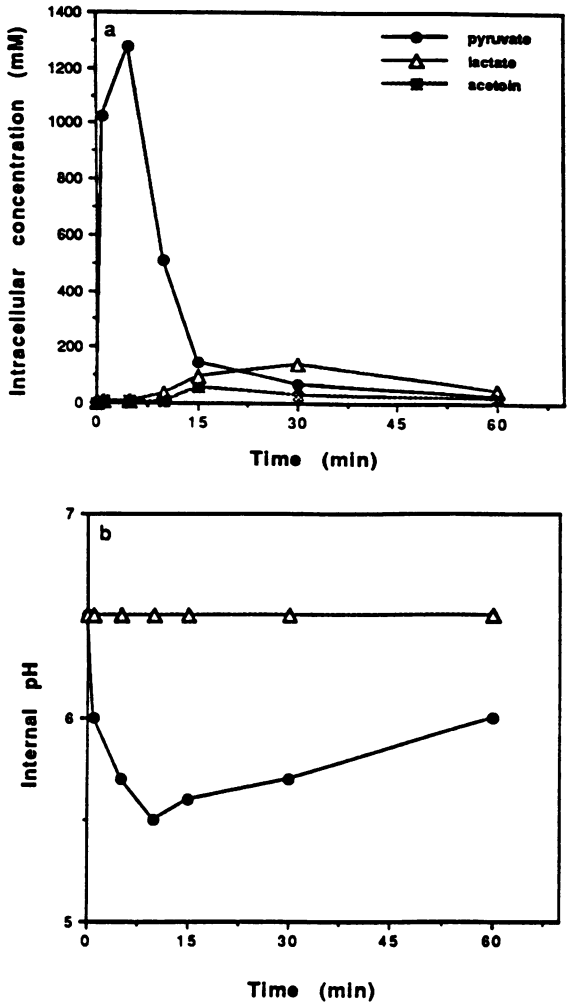

FIG. 3. (a) Transport of pyruvate and conversion of accumulated pyruvate into acetoin and lactate in *L. plantarum* cells undergoing glycolysis. Cells resuspended in 0.4 M potassium phosphate buffer (pH 4.5) were energized with 15 mM glucose for 15 min before ^{14}C-pyruvate was added at a final concentration of 20 mM. Radioactive pyruvate, lactate, and acetoin were separated by HPLC. (b) Effect of uptake and accumulation of pyruvate on pH homeostasis in energized *L. plantarum* cells. Symbols: ●, pyruvate added; Δ, in the absence of added pyruvate.

lular pyruvate pool where it mixed with glycolytically generated pyruvate and was partly converted to lactate. Acetoin did not accumulate intracellularly, since it is a neutral membrane-permeable compound. It was excreted, and almost all of the [^{14}C]acetoin was found in the supernatant after 1 h. At this point, 12.0 mM pyruvate had been utilized and converted into 2.7 mM lactate and 3.3 mM acetoin.

DISCUSSION

Most lactic acid bacteria maintain a constant PMF (16, 18). The PMF, which is generated by membrane-bound ATPase and end product efflux, is important for substrate transport (5, 12, 18, 20, 24). *L. plantarum* also maintained a constant PMF. However, the PMF value (−120 mV) was slightly lower than those found in *Lactococcus lactis* (16, 18), possibly because of the high potassium concentration in our buffer. Weak acid buffers could not be used because they are metabolized by *L. plantarum*.

It is well known that the addition of pyruvate enhances diacetyl and acetoin production (1–3, 8, 13). Pyruvate is actively transported in *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469 (1) and in *L. plantarum* where a proton symport is used (Fig. 3) (28). Thus, the size of the PMF, consisting primarily of ΔpH at low pH_e, drives pyruvate transport and consequently the amount of acetoin formed. The high pyruvate utilization rates and high conversion efficiency of pyruvate to acetoin at low pH_e in continuous culture (19) and resting cells (27) can now be attributed to the large pH gradient available for pyruvate uptake (Fig. 1a and b). The absence of glucose as an energy source resulted in a low ΔpH, little pyruvate transport, and no acetoin formation. In this study, we found that the protons cotransported with pyruvate decreased the pH_i by 1 pH unit. The conversion of the accumulated pyruvate to acetoin apparently contributed to pH homeostasis since the pH_i increased after the conversion. These results prove the hypothesis of Gunsalus and Harvey and Collins that high concentrations of intracellular pyruvate are necessary for acetoin formation. Conversion of pyruvate to acetoin is a mechanism of detoxification and pH homeostasis.

ACKNOWLEDGMENT

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