Journal of Industrial Microbiology, 4 (1989) 335–340
Elsevier

SIM 00191

pH-mediated regulation of pyruvate catabolism in *Lactobacillus plantarum* chemostat cultures

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Received 22 September 1988
Revised 14 December 1988
Accepted 22 December 1988

Key words: Acetoin; Lactate; Acetate; pH-homeostasis

SUMMARY

While the ability of lactobacilli to catabolize pyruvate to a variety of industrially important catabolites is well known, the mechanisms which regulate pyruvate distribution among alternative catabolic pathways is unclear. This paper demonstrates that environmental acidity regulates the catabolic activites of *Lactobacillus plantarum* cells in chemostat cultures. *L. plantarum* cells grown in medium containing 100 mM exogenous pyruvate, diverted pyruvate away from lactate to acetoin. Pyruvate uptake and acetoin generation increased under acidic conditions; on a molar basis, pyruvate utilization increased twice as fast as acetoin production, reflecting the 2:1 stoichiometry of pyruvate incorporation into acetoin. Lactate production increased under alkaline conditions when glucose was fermented to provide endogenous pyruvate. Acetate was formed only at pH 7.5 and 8.0, although acetoin production decreased at elevated pH values. These data indicate that *L. plantarum* adjusts to changes in environmental pH by altering its distribution of pyruvate among various catabolites.

INTRODUCTION

Pyruvate is the central intermediate in fermentative metabolism. The spectrum of industrially important catabolites produced by lactobacilli depends on how pyruvate is catabolized [17]. Homofermentative bacteria convert at least 85% of pyruvate formed from glucose catabolism to lactic acid [18]. This fermentation balance can be shifted to a heterofermentative pattern by varying medium compositions [9], culturing cells at low growth rates [7,32] and by exposing cells to oxygen [29].

In *Lactobacillus plantarum*, the major product of

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exogenous pyruvate catabolism is acetoin [10,16, 22]. Acetoin is formed through the condensation of 2 molecules of pyruvate to alpha-acetolactate which is subsequently decarboxylated to acetoin [16] (Fig. 1). The optimal pH for alpha-acetolactate and acetoin formation is 5.5 in *L. casei* [1]. Citrate, which is cleaved to acetate and a molecule of oxaloacetate that is decarboxylated to pyruvate, also stimulates acetoin and diacetyl production [10,16]. During fed-batch fermentations at pH 5.3, there are periods when pyruvate is quantitatively converted to acetoin. pH also influences the catabolism of endogenous pyruvate by homofermentative *Lactobacillus bulgaricus* [27]. Under alkaline conditions, pyruvate is diverted from lactate to acetate, formate and ethanol through the pyruvate-formate lyase pathway [11].

The objective of this study was to determine the regulatory influence of pH on pyruvate catabolism by *Lactobacillus plantarum* grown in continuous culture; a technique that controls variables such as growth rates [26], substrate concentrations [32] and cell density [33] which all influence catabolite production, but are impossible to control in batch culture [24]. We also report that homofermentative catabolism of glucose is regulated in response to environmental acidity.

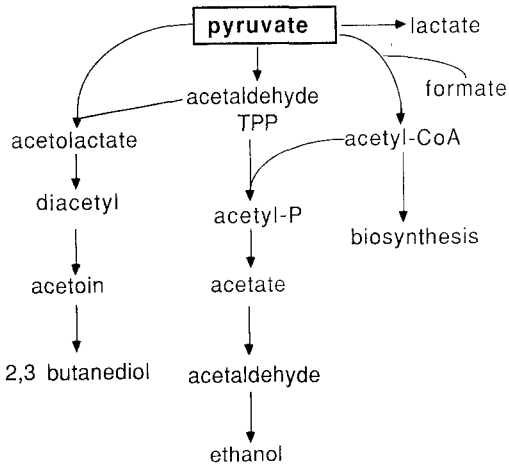

Fig. 1. Pathways for the catabolism of pyruvate in *Lactobacillus plantarum*.

MATERIALS AND METHODS

Strain

L. plantarum ATCC 8014, obtained from the American Type Culture Collection (Rockville, Maryland), was maintained in Lactobacillus MRS agar slants (Difco Laboratories, Detroit, Michigan) at 4°C and transferred monthly.

Culture Medium

The medium used was a modification of Craig and Snell [4] designated CS-T and consisted of (per liter of distilled water): K_2HPO_4, 600 mg; KH_2PO_4, 600 mg; $MgSO_47H_2O$, 200 mg; $MnSO_44H_2O$, 20 mg; $FeSO_47H_2O$, 10 mg; NaCl, 10 mg; Tween 80, 5 ml; Yeast Extract, 10 g. Pyruvate and glucose were sterilized separately and added to final concentrations of either 100 mM pyruvate plus 5 mM glucose [the glucose was added to provide energy for pyruvate transport (Tsau and Montville, Abs. Ann. Mtg. ASM, p. 267, 1988)] for studies on exogenous pyruvate; or 15 mM glucose to provide glycolytically- generated endogenous pyruvate. All chemicals were from Sigma Chemical Company (St. Louis, MO), except the yeast extract which was from Difco Laboratories (Detroit, MI).

Continuous Culture Conditions

A computer-controlled chemostat (Queue Systems, 'Mouse,' Parkersburg, West Virginia) with an 800 ml working volume was inoculated with 250 ml of an overnight culture which had been centrifuged and suspended in 10 ml of 0.9% saline. The chemostat was maintained at 35°C, dissolved O_2 at 0% (by a continuous overlay of nitrogen), and 250 rpm in a batch mode until the culture reached mid-exponential phase. Continuous feeding of fresh medium was then initiated at a dilution rate of 0.20 h^{-1}. The culture pH was controlled automatically by the addition of 0.5 N HCl or 0.5 N NaOH.

Analytical Methods

Samples were drawn when the chemostat reached steady state (i.e. after 5 residence times) at each pH value. Steady state was confirmed by the chemical analysis of at least three samples taken four hours

apart. All values reported here are the average of three steady state values which differed by $<10\%$. Cell dry weights were estimated by the method of Koch [19]. End-product and residual pyruvate concentrations were determined by high pressure liquid chromatography [25]. In some instances, lactate concentrations were also determined enzymatically by following the increase in NAD absorption at 340 nm. (Sigma Chemical CO). Chemostat effluents were tested for residual glucose using Diastix reagent strips (Miles Laboratories, Elkhart, IN) to verify glucose-limitation.

RESULTS AND DISCUSSION

Homofermentative bacteria catabolize glucose to pyruvate via the Embden-Meyerhoff-Parnas pathway [12]. The pyruvate is then reduced to lactate with the concomitant oxidation of NADH formed earlier in the pathway. When NAD-linked LDH (nLDH) is suppressed, homolactic microbes grow slowly. Therefore, LDH must be considered a key enzyme in energy production. In lactic streptococci and some lactobacilli, lactate is formed via a fructose-1,6-diphosphate activated, allosteric, nLDH [7,12,32]. In *L. plantarum*, nLDH is not allosteric [14] and is therefore not activated by fructose-1,6-diphosphate. *L. plantarum* can, however, as demonstrated below, produce diverse catabolic products.

Regulation of exogenous pyruvate catabolism

In the presence of 100 mM pyruvate and 5 mM glucose, *L. plantarum* 8014 reached a maximum for cell yield and lactate production at pH 5.5. (Fig. 2). This value is similar the optimum in pH-controlled fermentors [23]. Lactate formation is generally proportional to cell mass [22], perhaps because lactate excretion increases the membrane potential [30,31] contributing to the energy available for cell growth. Specific growth rates and cell yields are decreased when the lactate gradient is decreased [26]. Thus, while the chemostat cultures were presented with the same amount of substrate at all pH values, there was more energy available for biomass synthesis at pH 5.5.

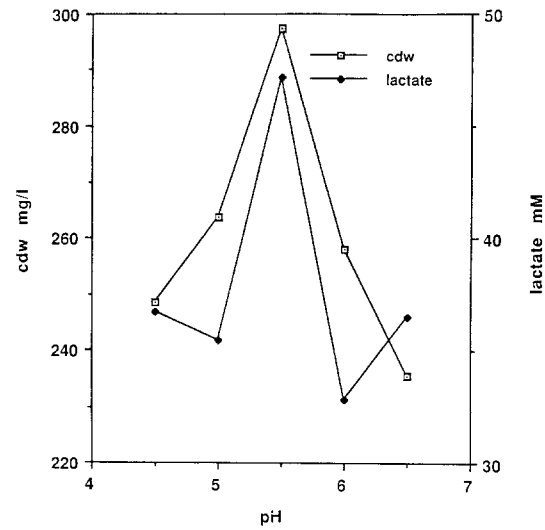

Fig. 2. The influence of environmental pH on production of cell dry weight (cdw) and lactate by *L. plantarum* 8014 grown in CS-T medium containing 100 mM pyruvate and 5 mM glucose at a dilution rate of 0.20 h^{-1} in a chemostat.

The uptake of pyruvate by *L. plantarum* 8014 is subjected to carbon catabolite repression, but energized by small quantities of glucose (Tsau and Montville, Abs. Ann. Mtg. ASM, p. 267, 1988). Low glucose concentrations also stimulate the concomitant production of acetoin from pyruvate [3].

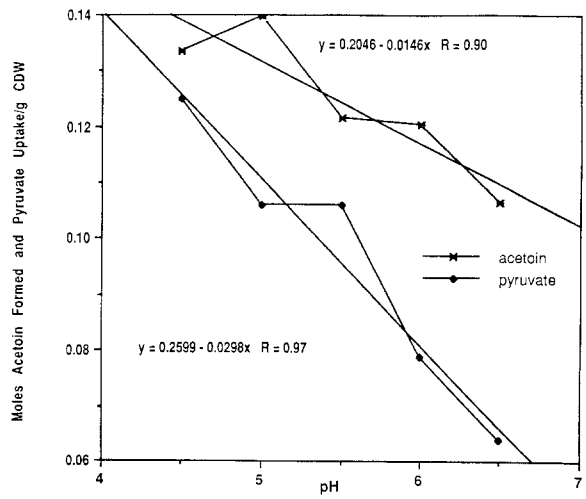

Fig. 3. The influence of environmental pH on pyruvate utilization and acetoin formation by *L. plantarum* 8014 grown in CS-T medium containing 100 mM pyruvate and 5 mM glucose at a dilution rate of 0.20 h^{-1} in a chemostat.

Glucose-dependent pyruvate transport and subsequent acetoin formation appear linked [5,10] by a mechanism currently under study in our laboratory. In the present experiments there was a linear relationship (r = 0.97) between pyruvate uptake and pH (Fig. 3). Under acidic condtions, more pyruvate entered the cell. This increase was most likely caused by two effects. Under acidic conditions, the greater ratio of protonated pyruvic acid to its ionized form, would increase pyruvate transport by facilitated diffusion. In addition, the transmembrane pH gradient would become higher with decreases in external pH.

L. plantarum 8014 also regulated acetoin production in response to environmental pH (Fig. 3). Acetoin formation was highest at pH 4.5–5.5, with a near linear decline at higher pH values. This is similar to results from pH-controlled fermentors [23] and test tube cultures [2,3], where acetoin synthesis is greatest at 5.0. The slope of the acetoin formation line (Fig. 3) was one half the slope of the pyruvate uptake line reflecting the reaction's stoichiometry. High levels of intracellular pyruvate may be required for acetoin formation [13]. The partially purified alpha-acetolactate from *Leuconostoc cremoris* is allosteric with at least 2 binding sites for pyruvate, inhibited by several products of glucose metabolism, but stimulated by low levels of glucose and its intermediates [3]. Its optimal pH is between 5 and 6. The formation of acetoin from alpha-acetolactate by *L. casei* has a similar pH optimum [1]. At the lower pH range, lactic acid bacteria produce more neutral compounds like acetoin, but the broad optimum for nLDH synthesis insures a homolactic fermentation under acidic conditions [27].

Regulation of endogenous pyruvate catabolism

In contrast to *L. bulgaricus* NLS-4 which, at neutral pH, shifts end products from lactate to a mixture of lactate, formate, acetate, and ethanol [27], *L. plantarum* 8014 made an increasing amount of lactate at higher pH values (Fig. 4). This discrepancy can be explained by comparing the organisms' pH optima for nLDH activities. It is 6.5 for *L. bulgaricus* but between 7.0 and 8.5 for *L. plantarum* [6,8,21].

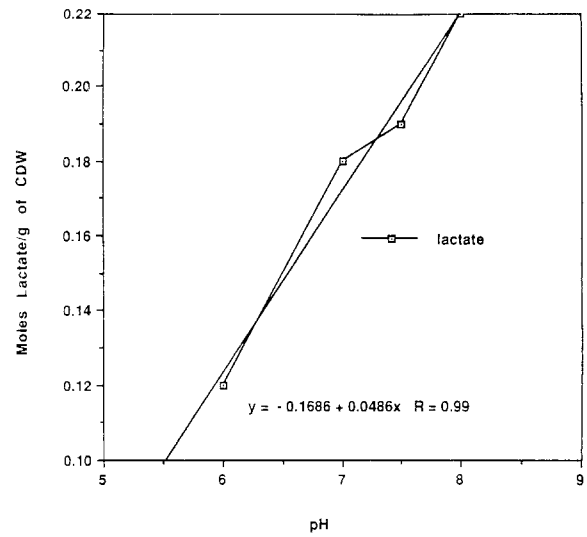

Fig. 4. The influence of environmental pH on lactate production by *L. plantarum* 8014 grown in CS-T medium containing 15 mM glucose at a dilution rate of 0.20 h^{-1} in a chemostat.

Acetoin production decreased with increasing pH (Fig. 5). This is similar to other lactic acid bacteria [3]. At more alkaline pH values, the acetoin-forming enzymes have to compete for intracellular pyruvate with pyruvate-formate lyase [21]. No acetate was detected at pH range $\leq$7.0. Acetate was pro-

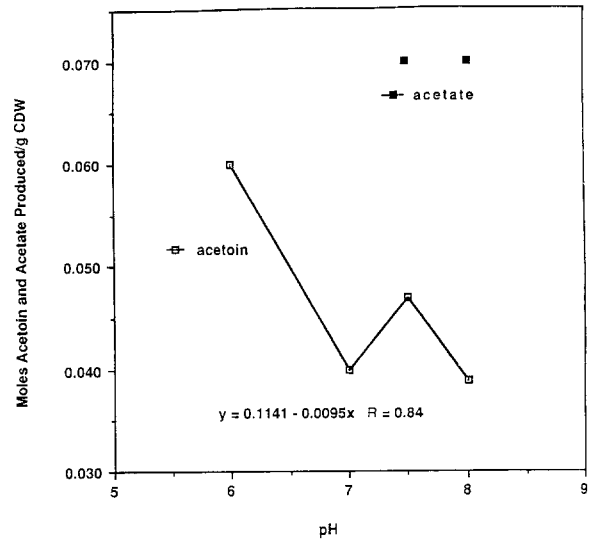

Fig. 5. The influence of environmental pH on acetoin and acetate production by *L. plantarum* 8014 grown in CS-T medium containing 15 mM glucose at a dilution rate of 0.20 h^{-1} in a chemostat.

duced at pH 7.5 and 8.0 at levels of 27.8 mM and 28.6 mM respectively (Fig. 5). Lactate concentrations also continued to increase (Fig. 4). The high pH optima for nLDH activity combined with the alkaline optima of the enzymes involved in the phosphoroclastic split: pyruvate-formate lyase reaction [20], phosphotransacetylase [15] and acetate kinase [28] explain why *L. plantarum* makes more acid at the expense of neutral compound in response to alkaline conditions.

The results presented here show that the 'homolactic' metabolism of *L. plantarum* is not invariable. The use of continuous culture enabled us to demonstrate that considerable quantities of acetoin can be made by growing cultures and that acetate can be produced under anaerobic conditions if the environment is alkaline. The ability to shift between the production of neutral and acidic compounds may help *L. plantarum* maintain pH homeostasis.

ACKNOWLEDGEMENTS

This is manuscript D-10112-2-88 of the New Jersey State Agricultural Experiment Station; supported by State appropriations and U.S. Hatch funds.

REFERENCES

1 Branen, A.L. and T.W. Keenan. 1970. Diacetyl, acetoin and 2,3-butanediol production by *Lactobacillus casei*. J. Dairy Sci. 53: 632.

2 Cogan, T.M., M. O'Dowd and D. Mellenick. 1981. Effect of pH and sugar on acetoin production from citrate by *Leuconostoc cremoris*. Appl. Environ. Microbiol. 41: 1–8.

3 Cogan, T.M., R.J. Fitzgerald and S. Doonan. 1984. Acetolactate synthase of *Leuconostoc lactis* and its regulation of acetoin production. J. Dairy Res. 51: 597–604.

4 Craig, J.A. and E.E. Snell. 1951. The comparative activities of pantethine, pantothenic acid and coenzyme A for various microorganisms. J. Bacteriol. 61: 238–291.

5 de Cardenas, J.L.B., O.V. Ledesma and G. Oliver. 1987. *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469. Current Microbiol. 15: 259–264.

6 Dennis, D. and N.O. Kaplan. 1960. D- and L-lactic acid dehydrogenases in *Lactobacillus plantarum*. J. Biol. Chem. 235: 810–818.

7 de Vries, W., W.M.C. Kapteyn, E.G. Van der Beek and Stouthamer. 1970. Molar growth yields and fermentation balances of *Lactobacillus casei* L3 in batch cultures and in continuous cultures. J. Gen. Microbiol. 63: 333–345.

8 Doelle, W.H. 1971. Nicotinamide adenine dinucleotide-dependent and -independent lactate dehydrogenases in homofermentative and heterofermentative lactic acid bacteria. J. Bacteriol. 108: 1284–1289.

9 El-Gendy, S.M., H. Abdel-Galil, Y. Shanin and F.Z. Heganzi. 1983. Acetoin and diacetyl production by homo- and heterofermentative lactic acid bacteria. J. Food Protect. 46: 420–425.

10 El-Gendy, S.M., H. Abdel-Gahil and F.Z. Hegazi. 1983. Acetoin and diacetyl production by *Lactobacillus plantarum* able to use citrate. J. Food Protect. 46: 503–505.

11 Fryer, F.F. 1970. Utilization of citrate by lactobacilli isolated from dairy products. J. Dairy Res. 37: 9–11.

12 Garvie, E.I. 1980. Bacterial lactate dehydrogenases. Microbiol. Rev. 44: 106–139.

13 Gunsalus, I.C. 1958. Energy metabolism of lactic acid bacteria, 4th Int. Cong. Biochem., Vienna 13: 444.

14 Hensel, R., U. Mayr, H. Fujiki and O. Kandler. 1977. Comparative studies of lactate dehydrogenases in lactic acid bacteria. Eur. J. Biochem. 880: 83–92.

15 Hibbert, F., S.A. Kyrtopoulos and D.P.N. Satchell. 1971. Kinetic studies with phosphotransacetylase. Biochim. Biophys. Acta 242: 39–54.

16 Hickey, M.W., A.J. Hillier and G.R. Jago. 1983. Metabolism of pyruvate and citrate in lactobacilli. Aust. J. Biol. Sci. 36: 487–496.

17 Ingraham, J.L., O. Maaloe and F.C. Nerdhadt. 1983. Polymerization, Biosynthesis, Fueling and Transport. Chapt. 3 In: Growth of the Bacterial Cell, pp. 136–150, Sinauer Associates, Inc., publishers, Sunderland, Mass.

18 Kandler, O. and N. Weiss, 1984. Regular, nonsporing, Gram-positive rods. Section 14 In: *Bergey's Manual of Systematic Bacteriology*, Vol. 2. (P.H.A. Sneath, ed.), Williams and Wilkens, Baltimore, MD.

19 Koch, A.L. 1981. Growth measurement. Ch. 11. In: (P. Gerhardt, R.G.E. Murray, R.N. Costilov, W.E. Nester, W.A. Wood, N.R. Krieg and G.B. Phillips eds.), Manual of Methods for General Bacteriology, p. 196. ASM, Washington. DC.

20 Lindmark, D.G., P. Paolella and N.P. Wood, 1969. The pyruvate formate-lyase system of *Streptococci faecalis*. J. Biol. Chem. 244: 3605–3612.

21 Mizushima, S. and K. Kitahara. 1962. Purification and properties of lactic dehydration of *Lactobacillus casei*. J. Gen. App. Microbiol. 8: 130–141.

22 Montville, T.J., M.E. Meyer and A.M.H. Hsu. 1987. Influence of carbon substrate on lactic acid, cell mass and diacetylacetoin production in *Lactobacillus plantarum*. J. Food Protect. 50: 42–46.

23 Montville, T.J., A.H.M. Hsu and M.E. Meyer. 1987. High-efficiency of pyruvate to acetoin by *Lactobacillus plantarum*

during pH-controlled and fed-batch fermentations. Appl. Environ. Microbiol. 53: 1798–1802.

24 Montville, T.J. 1987. Continuous culture: theory and applications. In: (Montville, T.J., ed.), Food Microbiology Vol. 2: New and Emerging Technologies. p. 165–186, CRC Press, Boca Raton, GL.

25 Montville, T.J., A.H.M. Hsu, M.E. Meyer and G.T.C. Huang. 1987. High pressure liquid chromatography and wide-bore capillary gas chromatography methods for acetoin and diacetyl. J. Microbiol. Meth. 7: 1–8.

26 Otto, R., B. ten Brinks, H. Veldkamp and W.N. Konings, 1983. The relation between growth rate and electrochemical proton gradient of *Streptococci cremoris*. FEMS Microbiol. Lett. 16: 69–74.

27 Rhee, S.K. and M.Y. Pack. 1980. Effect of environmental pH on fermentation balance of *Lactobacillus bulgaricus*. J. Bacteriol. 144: 217–221.

28 Satchell, D.P.N. and G.F. White. 1970. Kinetic studies with acetate kinase. Biochim. Biophys. Acta 212: 248–256.

29 Smart, J.B. and T.D. Thomas. 1987. Effect of oxygen on lactose metabolism in lactic streptococci. App. Environ. Microbiol. 53: 533–541.

30 ten Brinks, B. and W.N. Konings. 1982. Electrochemical proton gradient and lactate concentration gradient in *Streptococcus cremoris* cells grown in batch culture. J. Bacteriol. 152: 682–686.

31 ten Brinks, B., R. Otto, U.P. Hansen and W.N. Konings, 1985. Energy recycling by lactate efflux in growing and non-growing cells of *Streptococci cremoris*. J. Bacteriol. 162: 383–390.

32 Thomas, T.D., D.C. Ellwood and U.M.C. Longyear. 1979. Change from homo- to heterofermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. J. Bacteriol. 138: 109–117.

33 Thomas, T.D. and K.W. Turner. 1981. Carbohydrate fermentation by *Streptococcus cremoris* and *Streptococcus lactis* growing in agar gels. Appl. Environ. Microbiol. 41: 1289–1294.