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Anaerobic L-lactate degradation by *Lactobacillus plantarum*

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1. SUMMARY

Lactobacillus plantarum strains used as silage inoculants were investigated for their ability to metabolize lactic acid anaerobically after prolonged incubation (7–30 days) when glucose was absent from the medium. When citrate was present in the medium together with glucose during the initial fermentation, the lactic acid produced was degraded. Citrate was concomitantly degraded, resulting in accumulation of formic, acetic and succinic acids along with CO_2. The anaerobic degradation was confirmed by the use of L-^{14}C(U) labelled lactate. The existence of pyruvate formate lyase in *L. plantarum* was indicated by using ^{14}C-labelled pyruvate and HPLC identification of end-products. The 1-^{14}C-carboxylic acid group of pyruvate was converted to formic acid, and the 3-^{14}C was found in acetic acid. The key enzyme(s) in this metabolic pathway appears to require anaerobic conditions and induction by citrate.

2. INTRODUCTION

The general assumption that *Lactobacillus plantarum* ferments hexoses primarily to lactic acid has been revised. Results obtained during the last decade indicate a considerable variation in end-product formation, depending on substrate and cultivation conditions. Traces of acetate, acetoin, diacetyl, 2,3-butanediol, and ethanol have been identified during prolonged incubation on glucose [1,2]. High concentrations of pyruvate can be converted anaerobically to lactic acid and acetyl-phosphate [3]. Pyruvate also stimulates production of acetoin [4].

In silages inoculated with starter cultures containing *L. plantarum*, we have observed a degradation of lactic acid with a simultaneous increase in acetic acid concentration after prolonged storage. The degradation of lactate to acetate requires an external electron acceptor. Aerobically, oxygen can serve this purpose [5], but the anaerobic conditions in silage excludes this possibility as an

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explanation for our observation. McFeeters and Chen [6] have shown that citrate can be used as an electron acceptor for the anaerobic degradation of mannitol by *L. plantarum*, resulting in succinate and ethanol formation in addition to lactate.

The objectives of this study were to prove the existence of a pathway for anaerobic degradation of lactic acid and to determine conditions which affect the metabolic pathway.

3. MATERIALS AND METHODS

3.1. Organisms

Five strains of *L. plantarum* were isolated from five different inoculants used for commercial silage making (Siloferm, AB Milkfood, Kagerod, Sweden; Biomax, Chr. Hansen, Copenhagen, Denmark; Lactisil, Medipharm, Angelholm, Sweden; Natuferm, Apotekarnes AS, Oslo, Norway; Miles Labs, Elkhart, Indiana, U.S.A.). The strains were isolated on MRS agar (Oxoid) and identified as *L. plantarum* based upon sugar utilization with API 50 CH (API System, Montalieu-Vercieu, France) and the presence of meso-diaminopimelic acid in the cell wall [5]. They were kept deep-frozen in Lactobacillus Caring Medium (LCM) [7] containing 15% glycerol. LCM without citrate was used as the basal medium during the initial experiment. The complete synthetic medium (CSM) described by McFeeters and Chen [6] was used for a more exact evaluation of end-product formation and metabolic characteristics.

3.2. Culture conditions with nonlabelled substrates

Culture flasks (100 ml) were filled with 80 ml of either medium and supplemented with citrate, glucose or L-lactate at different concentrations. The media were adjusted to pH 5.0 with 5 M NaOH or 5 M HCl, and the flasks were sealed with rubber stoppers. Anaerobic conditions were created by evacuating the air and replacing with 100% sterile nitrogen gas. Flasks containing LCM were autoclaved 15 min at 121°C. Flasks with CSM were boiled for 5 min and then held at 117°C for 1 min. Strains to be used as inocula were pre-grown for 2 days at 28°C in a small volume of LCM or CSM, both supplemented with 10 mM glucose. Cultures were incubated with gentle shaking at 28°C, with samples taken regularly using sterile syringes, and stored at −20°C until analyzed. For the adaptation experiment, cells from 2-week old flask cultures (80 ml), containing CSM (plus 10 mM glucose) and with or without citrate (10 mM), were harvested by centrifugation, washed, resuspended in a small volume of sterile water, and transferred to new flasks containing 80 ml of CSM with 15 mM L-lactate and 10 mM citrate. Incubation was performed anaerobically as described above.

3.3. Culture conditions with labelled substrates

The experiments were performed in 25 ml flasks. The flasks were stored in an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, MI) and sealed with a rubber stopper. Samples were taken by a sterile syringe. Anaerobic L-lactate degradation was evaluated in 5 ml of CSM sterile-filtered through a 0.20 μm membrane (Costar, Cambridge, MA). The medium contained 10 mM glucose, 10 mM citrate, 10 μl lactic acid, sodium salt, L-[^{14}C(U)] dissolved in 70% ethanol (NEN Research Products, DuPont). The labelled lactic acid solution contained 0.56 μmol acid ml^{-1} and 0.1 mCi radioactivity ml^{-1}. The medium was inoculated with strain 5 as described above. Pyruvate degradation was determined in 5 ml of sterile-filtered CSM containing 50 mM pyruvate. Twenty μl pyruvic acid, sodium salt, [1-^{14}C] or [3-^{14}C] (NEN) dissolved in 0.2 M HCl was added to 5 ml CSM. The 1-^{14}C isotope had a concentration of 1 μmol acid and 0.05 mCi radioactivity ml^{-1} and the 3-^{14}C isotope 2.9 μmol acid and 0.05 mCi radioactivity ml^{-1}, respectively. Each medium was inoculated with cells of strain 5 from an equal amount of culture liquid adapted in citrate and glucose as described above.

3.4. Metabolite analysis of nonlabelled samples

Organic acids and ethanol were measured using high pressure liquid chromatography (HPLC). The samples were centrifuged at 15000 × *g* (Eppendorf tubes). The supernatant fractions were diluted in 0.1% isovaleric acid and injected (10 μl) into the HPLC apparatus with a Milton Roy pump, Aminex HPX-87H column at 45°C and

Tecator Optilab 5902 refractometer with a 10 mm cell. The column was eluted with 0.005 M H_2SO_4 at a flow rate of 0.4 ml min^{-1}. Peak areas were calculated using a computer integrator (Spectra-Physics 4270). External standards were citric acid, malic acid, succinic acid, lactic acid, formic acid, acetic acid, ethanol, and isovaleric acid. Occasionally, acetoin, diacetyl and glucose were added to the standard. Isovaleric acid also served as an internal standard. CO_2 was analyzed in a gas chromatograph (Carlo Erba, 230) equipped with a hot wire detector. The column was a Porapak QS (mesh 80–100) with a diameter of 6 mm. The oven temperature was 60 °C and the detector temperature was 150 °C. Helium was used as the carrier gas with a constant flow of 30 ml min^{-1}.

3.5. Metabolite analysis of labelled samples

Samples with labelled lactic acid or pyruvic acid were taken after 4 weeks' storage. A 1-ml sample was centrifuged in an Eppendorf centrifuge. A 20 µl sample was injected onto a Bio-Rad HPX-87H HPLC column with a Bio-Rad cation exchange guard column. The column was eluted with 0.005 M H_2SO_4 at a flow rate of 0.5 ml/min. Compounds were detected using a Waters model 401 refractive index (RI) detector. The effluent from the RI detector was collected manually at either 15 or 30 s intervals into scintillation vials. The CPM were measured in a Beckman scintillation counter after addition of 4.5 ml scintillation liquid (Scintiverse E, Fisher Scientific Co.) to each vial.

4. RESULTS

In the first experiment, metabolic activity for five strains of *L. plantarum* isolated from five different commercial silage inoculants were evaluated anaerobically in LCM broth enriched with 20 mM glucose and 10 mM citrate. All strains reduced the citrate and lactate concentration and accumulated acetate after prolonged (7–30 days) incubation. All strains, however, produced higher concentrations of acetate, than could be accounted for by the sugar added to LCM. Therefore, a complete synthetic medium (CSM) was used for subsequent evaluation of metabolic characteristics.

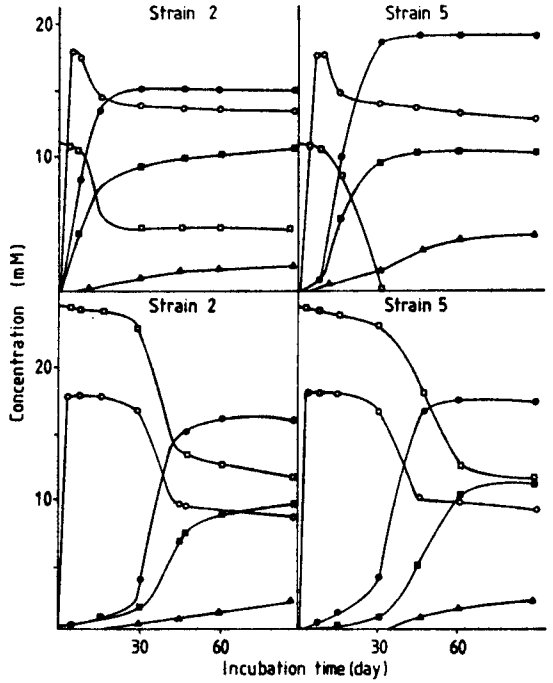

Fig. 1. Changes in fermentation products of two strains of *L. plantarum* in complete synthetic medium containing 10 mM glucose and 10 mM citrate (top) or 26 mM citrate (bottom). □, citrate; ○, lactate; ●, acetate; ■, formate; ▲, succinate. Carbon dioxide was identified in the gas phase of each flask.

Metabolic activities for strains 2 and 5 were further evaluated by analysis after prolonged incubation in CSM with different substrates. Citrate was not metabolized. Glucose was fermented to lactate when it was added alone to CSM. If the medium was supplemented with both citrate and glucose, glucose was again fermented to lactate, but subsequently the citrate and lactate were degraded. The following metabolic end-products were identified: succinic acid, lactic acid, formic acid, acetic acid, and carbon dioxide (Fig. 1). Malate was observed as an intermediate. Acetoin and diacetyl were not produced. More lactate was degraded as the concentration of citrate in the medium was increased. Aerobically, the citrate was not affected, and the sugar was simultaneously metabolized to lactate and acetate.

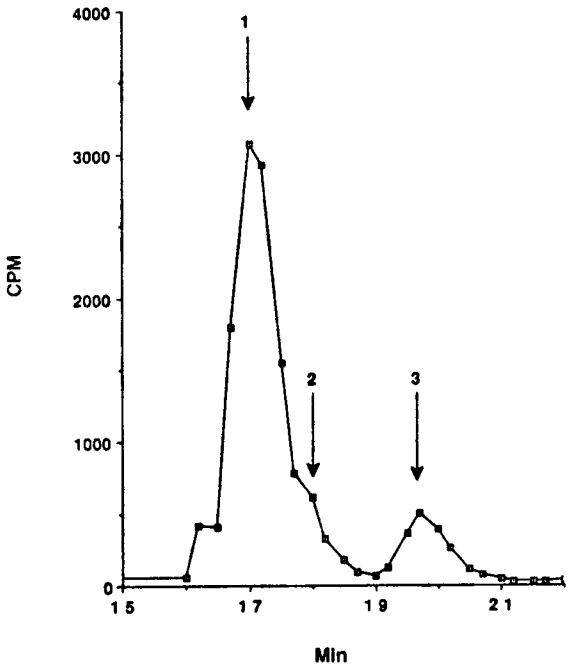

Fig. 2. Radioactivity (CPM) in HPLC fractionated samples of anaerobic degraded lactic acid, L-^{14}C (U) after 4 weeks' growth by *L. plantarum*. Arrows (↓) indicate the mean retention time for: (1), lactic acid; (2), formic acid; (3), acetic acid.

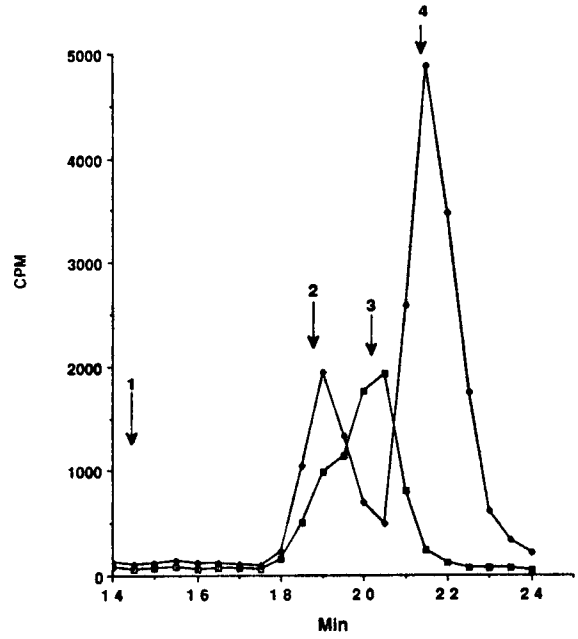

Fig. 3. Radioactivity (CPM) in HPLC fractionated samples of anaerobic degraded pyruvate after 4 weeks' growth by *L. plantarum*. □, pyruvate labelled in 1-^{14}C position; ◆, pyruvate labelled in 3-^{14}C position. Arrows (↓) indicate the mean retention time for: (1), pyruvic acid; (2), lactic acid; (3), formic acid; (4), acetic acid.

The anaerobic degradation of L-lactate to acetate and formate was confirmed by degradation of L-^{14}C(U) labelled lactate (Fig. 2). An acetate peak was separated from lactate. The formate peak could not clearly be separated from the lactate due to the small difference in retention time. The formate radioactivity could, however, be observed as a shoulder on the lactate peak.

Table 1

Metabolite concentration after 30 days of anaerobic incubation in CSM with 15 mM L-acetate and 10 mM citrate with *L. plantarum* strain 5. The strain was first adapted for 2 weeks in CSM + 10 mM glucose with or without citrate (10 mM).

Metabolite	Adaptation without citrate		Adaptation with citrate	
	Experiment 1 (mM)	Experiment 2 (mM)	Experiment 1 (mM)	Experiment 2 (mM)
Citrate	10	10	0	0
Succinate	0	0	3	3
Lactate	15	15	11	11
Formate	0	0	7	9
Acetate	0	0	20	21
Ethanol	0	0	0	0

Table 1 shows that *L. plantarum* strain 5 required an adaptation period in glucose-citrate medium to degrade lactate. Cells pre-grown in medium with only glucose did not affect the lactate or citrate concentrations.

The existence of a pyruvate formate lyase was indicated by the degradation of ^{14}C-labelled pyruvate. Three-foruths of the radioactivity in 3-^{14}C pyruvate was localized to the acetic peak. The remaining was found in the lactic acid peak. No radioactivity was found at the retention time of pyruvate (Fig. 3). The main radioactivity after degradation of 1-^{14}C-pyruvate was found in the formate peak (Fig. 3). A small amount of activity was also found in the lactate peak.

5. DISCUSSION

In the absence of fermentable sugars, strains of *L. plantarum* are able to metabolize lactate anaerobically with the formation of formate and acetate. The activity was observed initially in a racemic mixture of D- and L-lactic acid produced by *L. plantarum* in a glucose-containing substrate. Though the degradation was confirmed by the use of L-^{14}C(U) lactate, this metabolic activity is likely to exist for the D-isomer as well. This metabolic activity requires the presence of citrate in the medium. Citrate, or rather oxaloacetate formed from citrate by the action of citrate lyase, has been shown to act as electron acceptor for the anaerobic mannitol fermentation by *L. plantarum* [6], resulting in succinate formation. In the lactate/citrate metabolism, succinate is also formed (Fig. 2), indicating that the oxaloacetate derived from citrate is used as an electron acceptor. NADH is probably formed during the oxidation of lactate to pyruvate. However, the ratio between mols of succinate formed to citrate used was less than 1.0, indicating that oxaloacetate may also be used for the formation of pyruvate, presumably through decarboxylation since carbon dioxide was evolved. Decarboxylation of oxaloacetate is common in lactic acid bacteria in connection with diacetyl or acetoin formation from citrate [3].

In our experiments, pyruvate is hypothetically formed both from lactate and oxaloacetate. This accumulation did not induce a diacetyl-acetoin metabolism similar to that reported by Montville et al. [4]. Instead, our results suggested the operation of a pyruvate-formate lyase resulting in formate and acetate production. This enzyme is characteristic for enterobacteria [3], but has also been observed in streptococci [7–9] and *Lactobacillus casei* [10] under anaerobic conditions. This is, to our knowledge, the first observations indicating the existence of pyruvate-formate lyase activity in *L. plantarum*. The action of pyruvate-formate lyase probably drives the lactate oxidation to pyruvate by lactate dehydrogenase, a reaction for which the equilibrium normally lies in favor of lactate.

Apparently, some key enzyme(s) must be induced by the presence of citrate during growth, since cells pre-grown in glucose without citrate were unable to degrade lactate. Citrate lyase was shown to be constitutive in a strain of *L. plantarum* [11]. The enzyme activity increased 3-fold when citrate was present in the medium during growth on glucose-citrate medium as compared to glucose medium.

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