Utilization of Lactic Acid by *Fusarium oxysporum* var. *lini*: Regulation of Transport and Metabolism

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Lactic acid was transported in *Fusarium oxysporum* var. *lini* ATCC 10960 by a saturable transport system that had a half-saturation constant of 56.6 ± 7.5 μM and a maximum velocity of 0.61 ± 0.10 mmol h⁻¹ g⁻¹ (dry weight) at 26°C and pH 5.0. This transport system was inducible and was not expressed in the presence of a repressing substrate. Evidence is presented that the anionic form lactate⁻ was taken up by the cells. Propionic, acetic, pyruvic, and bromoacetic acids but not succinic acid competitively inhibited the transport of lactic acid. Bromoacetic acid, which was not metabolized, was taken up to a steady-state level when intracellular and extracellular concentrations were identical, indicating that the transport system was not accumulative. The enzymatic activity that was physiologically more relevant in the metabolism of lactic acid was lactate: ferricytochrome c oxidase. This enzyme did not exhibit stereospecificity and was induced by lactic acid.

The ability to utilize lactic acid as an energy and carbon source is common among microorganisms, but the mechanisms of uptake in eukaryotes have been known for just a few yeasts. H⁺-lactate symports in *Candida utilis* (5, 9) and *Saccharomyces cerevisiae* (3) have been described, and a lactate unipor in *Kluyveromyces marxianus* has also been described (6).

Two types of enzymatic activities that are directly involved in the oxidation of lactate have been found in yeasts: lactate:ferricytochrome c oxidoreductases (E.C. 1.1.2.3 and E.C. 1.1.2.4, which are specific for D- and L-lactate, respectively) (12) and lactate:NAD oxidoreductase (E.C. 1.1.1.28 or E.C. 1.1.1.27, which catalyzes the oxidation of D or L-lactate) (10).

It has been suggested that *Fusarium oxysporum* may be a producer of single-cell protein whose nutritive quality is high (15). In Brazil, where vinasse, a by-product of the distillation of fuel ethanol, is a serious pollution problem, a big effort is being made to try to utilize the nutrients present in vinasse as resources for the production of single-cell protein. The results of studies on the nutritive value of the protein produced by using vinasse as a raw material have indicated that a good-quality food might be produced when *Fusarium oxysporum* var. *lini* is grown on this substrate (13). In previous work data on the uptake of sugars (2) and glycerol (4) have been reported. These compounds, together with lactic acid, are the main organic constituents of vinasse; they are the main source of energy and carbon for production of single-cell protein and/or the main pollutants (biological oxygen demand) in vinasse.

In this paper we describe some properties of lactic acid uptake and metabolism in *F. oxysporum* var. *lini* and describe the conditions under which these processes occur in relation to the metabolism of glucose and glycerol.

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**MATERIALS AND METHODS**

**Organism**. *F. oxysporum* var. *lini* ATCC 10960 was kept on potato-dextrose agar at 4°C after growth for 5 days at 30°C.

**Growth and harvesting conditions**. In order to study growth parameters, the fungus was grown in a medium containing 1% peptone, 0.2% yeast extract, 0.5% KH₂PO₄, 0.075% MgSO₄ · 7H₂O, and the carbon sources indicated below.

The medium used to grow cells for transport and enzyme assays contained 0.5% (NH₄)₂SO₄, 0.5% KH₂PO₄, 0.05% MgSO₄ · 7H₂O, 0.013% CaCl₂, 2H₂O, oligoelements, and vitamins (14). To grow cells, 200-ml portions of the appropriate medium were placed in 1-liter shake flasks that were kept in an orbital incubator at 26°C. The cells were harvested in the mid-exponential phase by filtration on glass fiber membranes (Whatman type GF/C) and washed with deionized water at room temperature. The cells used for transport and enzyme assays were kept on ice for no more than 1 h.

**Transport measurements**. To measure initial rates of uptake of labelled lactic acid and H⁺ and K⁺ movements, we used previously described methods (11). To evaluate the initial rate of uptake of the labelled acid, a preparation containing 20 μl of cell suspension (=0.6 mg [dry weight]), 20 μl of buffer (100 mM Tris–MES [morpholineethanesulfonic acid], pH 5.0), and 10 μl of a solution of Dl-[1-¹⁴C] lactic acid (specific activity, 50 Bq nmol⁻¹ for concentrations up to 0.2 mM and 17 Bq nmol⁻¹ for higher concentrations) was incubated at 26°C for 10 s. Incorporation was stopped by adding 5 ml of ice-cold water. The cells were immediately filtered and washed on Whatman type GF/C membrane filters, and radioactivity was counted by using a liquid scintillation system. Controls were prepared by adding 5 ml of cold water prior to the addition of the labelled solution. To check that the measurements were good estimates of initial uptake rates, the linearity of incorporation with time was confirmed for periods up to 20 s. All assays were performed in duplicate. Inhibition of initial lactic acid uptake by other monocarboxylic acids was determined by using the same procedure and adding monocarboxylic acids at appropriate concentrations to the buffer. Incorporation of the radioactive compound was initiated by adding cells. DL-[1-¹⁴C] lactic acid was obtained from Amersham. The

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movements of ions (H\(^+\) and K\(^+\)) associated with the initial uptake of lactic acid were evaluated by monitoring the external ion concentrations in thick suspensions of cells as described previously (11).

**Measurement of cellular volume.** To measure the volume of lactic acid-grown cells, we used a previously described method (4) in which \(^{60}\)Co-EDTA was used as an extracellular marker. A value of 2.16 \(\pm\) 0.22 \(\mu\)l mg (dry weight)\(^{-1}\) was determined.

**Accumulation ratios.** Accumulation ratios were calculated for \([1\text{-}1^4\text{C}]\)propionic acid and for bromo-[1\text{-}1^4\text{C}]\)acetic acid by using specific activities of about 50 Bq/nmol. The measurements were determined at 26°C in small test tubes with magnetic stirring. Each tube contained 500 \(\mu\)l of cell suspension and 500 \(\mu\)l of 100 mM Tris buffer adjusted to pH 5 with MES. At zero time 100 \(\mu\)l of labelled solution was added to give a final concentration of 0.5 mM. At appropriate times 100-\(\mu\)l samples were removed, filtered, and washed, and radioactivity was counted by using a liquid scintillation system. Intracellular concentrations were calculated by taking into account the internal volume indicated above.

**Substrate concentrations in growth media.** The concentrations of glycerol and glucose in culture media were estimated enzymatically by using a Boehringer Biochimica test combination for neutral fat and glycerol and a glucose oxidase-peroxidase kit (Boehringer), respectively. The lactic acid concentration was estimated with a L-(+)-lactate determination kit (Sigma Chemical Co., St. Louis, Mo.). The concentrations of lactate and lactic acid were calculated by using the Henderson-Hasselbalch equation and assuming a pH value of 3.86 (16). Samples were prepared by centrifugation.

**Preparation of cell extracts.** We used a version of the method described by Funayama et al. (7) to prepare cell extracts. Pellets containing about 0.3 g (wet weight) of fresh cells were frozen at \(-20^\circ\)C in 10-ml test tubes. After 0.5 ml of the appropriate buffer was added to each enzyme assay mixture and after 1.2 g of glass beads (diameter, 0.5 mm) was added, the tubes were vigorously shaken four times (1 min each) with a laboratory tube shaker (Vortex Jr. mixer) with intervals of 1 min on ice. After 1 ml of the same buffer was added, the cell homogenates were transferred into micro test tubes and centrifuged at 6,000 rpm twice for 4 min. The supernatants were used for enzyme assays.

**Enzyme assays and protein determination.** The activities of enzymes were assayed at 30°C with a Perkin-Elmer model 552 spectrophotometer connected to a Perkin-Elmer model 561 absorbance recording system.

L- or D-Lactate:ferredoxin oxidoreductase activity was assayed as described by Labeyrie et al. (8) at 420 nm by using potassium ferricyanide as the electron acceptor in a reaction mixture containing 100 mM Na\(^+\) phosphate buffer (pH 7.0), 1.0 mM EDTA, and 1.0 mM potassium ferricyanide. The reaction was started by adding D- or L-lactate in the same buffer after the endogenous reduction of ferricyanide was monitored for 3 min.

The method of LeJohn and Stevenson (10) was used to measure the activity of D- or L-lactate:NAD oxidoreductase at 340 nm in a reaction mixture containing 100 mM Tris adjusted with acetic acid to pH 8.0 and 0.2 mM NADH. The reaction was started by adding 100 mM pyruvate. The rate of NADH oxidation prior to substrate addition was used as the control rate. Enzyme activities, expressed in international units, were calculated by using the following absorption coefficients: 6.22 mM\(^{-1}\) cm\(^{-1}\) for NADH and 1.04 mM\(^{-1}\) cm\(^{-1}\) for ferricyanide.

The protein contents of crude extracts were estimated by the method of Bradford (1) at 495 nm by using bovine serum albumin as the standard. Enzymes and substrates were obtained from Sigma. All reagents were analytical grade.

**RESULTS**

**Growth on lactic acid.** *F. oxysporum* was able to grow on lactic acid as a sole energy and carbon source. The maximum specific growth rate was 0.08 h\(^{-1}\), and the yield coefficient was 29%. Growth was identical with L-lactic acid and DL-lactic acid.

When the medium contained 0.5% lactic acid, and 0.5% glucose, diauxic growth was observed; the glucose was consumed before the lactic acid was consumed. When 0.5% glycerol was added to the same medium, the same diauxic behavior was observed; the glucose was utilized first, but the lactic acid and glycerol were consumed simultaneously (Fig. 1).

**Transport.** The kinetics of the initial rate of uptake of DL-lactic acid were investigated for the concentration range from 0.025 to 20 mM by using DL-lactic acid-grown cells. Our results indicate that saturation kinetics were involved; a half-saturation constant \((K_s)\) of 56.6 \(\pm\) 7.5 \(\mu\)M and a maximum velocity of 0.61 \(\pm\) 0.10 mmol h\(^{-1}\)g (dry weight)\(^{-1}\) were calculated when experiments were performed at 26°C and pH 5.0. (The results indicated are the means \(\pm\) standard deviations from four independent experiments.)

A number of related compounds were tentatively tested as competitive inhibitors of lactic acid transport; propionic acid \((K_i, 6.9 \text{ mM})\), acetic acid \((K_i, 0.1 \text{ mM})\) but not succinic acid competitively inhibited the transport of lactic acid. Figure 2 shows the inhibition of lactic acid transport by propionic acid.

The effect of external pH on the kinetic parameters was investigated in order to clarify which form, the anionic form or the protonated form, was transported through the plasma membrane. The results are summarized in Table 1. The initial uptake of DL-lactic acid was measured in two independent experiments at external pH values of 4.0, 5.0, and 6.0. The \(K_s\) values were calculated by taking into consideration the concentration of each chemical species of the compound at the pH used. If the protonated form was actually transported, the \(K_s\) value varied about 50-fold in the range from pH 4 to pH 6; if the ionized form was transported, the \(K_s\) value varied only slightly in the same pH range. This is a good indication that the system is probably specific for lactate.

In an attempt to elucidate the mechanism involved in the transport of lactate, we monitored H\(^+\) and K\(^+\) movements associated with the initial uptake of the anions. No appreciable changes in the external concentrations of these ions were observed when a pulse consisting of 1 mM lactic acid was added to a cell suspension, indicating that no specific movements of these ions were associated with the uptake of lactate. The hypothesis that charge might be balanced by an anion antiport was not examined.

Propionic acid, a competitive inhibitor of lactate transport which did not support growth, was the first compound that we tried as an analog in order to check the accumulative nature of the transporter. Surprisingly, after rapid and strong accumulation of radioactive acid inside the cells, all of the radioactivity was quickly lost from the suspension, indicating that, although propionic acid did not support growth, it was metabolized by both lactic acid- and glucose-grown cells. After we established that lactic and bromoacetic acids
were mutual competitive inhibitors of transport, we evaluated the ability of lactic acid-grown cells to accumulate [1-14C]bromoacetic acid. A steady state was reached when intracellular and extracellular concentrations were identical (the accumulation ratio was about 1.5), indicating that the transport system was not accumulative. Neither 1 mM lactic acid nor 50 μM carbonyl cyanide m-chlorophenylhydrazone elicited the exit of labelled compound.

**Regulation of lactic acid transport.** The measurements of uptake rates described above were obtained by using cells grown under conditions in which lactic acid was the sole energy and carbon source. Cells grown on glucose, on glucose plus lactic acid, or on ethanol did not transport lactic acid, indicating that the gene(s) is regulated by induction and glucose repression and not expressed in the presence of a repressing substrate.

An interesting observation was made: during growth of submerged cultures on lactic acid at some stage massive production of conidia occurred. Concomitantly, the lactic acid transport activity decreased to values near zero.

**Enzyme activities and their regulation.** In order to enhance our understanding of lactic acid metabolism and enzyme regulation in *F. oxysporum* var. *lini*, we measured the activities of the enzymes presumably involved in lactic acid metabolism in extracts of cells grown under different conditions. The specific activities of these enzymes are shown in Table 2. Our results indicate that the enzymatic activity that is physiologically more relevant is certainly the lactate:ferricytochrome c oxidase activity that is responsible for the conversion of lactate to pyruvate in the fungus. This enzyme did not exhibit stereospecificity and was induced by lactate. A lactate:NAD-oxidoreductase activity was found in extracts of lactate-grown cells but was not detected in cells grown under other conditions. This enzyme catalyzed the reduction of pyruvate to lactate but not the reverse reaction.

**DISCUSSION**

Our results constitute evidence that lactic acid is transported by a carrier through the plasma membrane of *F. oxysporum* var. *lini*.

![FIG. 1. Diauxic growth of *F. oxysporum* var. *lini* in rich medium containing 0.5% glucose, 0.5% glycerol, and 0.5% lactic acid. Symbols: ○, biomass; ■, □, and △, concentrations of glucose, lactic acid, and glycerol in the medium, respectively.](image1)

![FIG. 2. Lineweaver-Burk plots of initial rates of uptake at pH 5.0 of labelled d- lactic acid by lactic acid-grown *F. oxysporum* var. *lini* cells in the absence of propionic acid (■) and in the presence of 1 mM (□), 5 mM (●), and 20 mM (○) propionic acid.](image2)

**TABLE 1. Kinetic constants for the transport of the ionized and protonated forms of dL-lactic acid in lactic acid-grown cells of *F. oxysporum* var. *lini* as a function of pH as determined in two independent experiments.**

<table>
<thead>
<tr>
<th>Expt</th>
<th>pH</th>
<th>$K_c$ (LAC-)$^*$ (μM)</th>
<th>$K_c$ (HLAC) (μM)</th>
<th>Maximum velocity (mmol g⁻¹ h⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>6.0</td>
<td>66.4</td>
<td>0.48</td>
<td>5.91</td>
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</tr>
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<td>4.0</td>
<td>50.1</td>
<td>36.31</td>
<td>6.01</td>
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</table>

$^*$ $K_c$ values were estimated from Lineweaver-Burk plots of the initial uptake of labelled dL-lactic acid by assuming that either the protonated form (HLAC) or the anionic form (LAC⁻) of the acid was accepted by the carrier.
oxysporum var. lini. Inhibition by other weak acids might be caused by a nonspecific effect based on intracellular acidification which might affect the affinity of lactate for the carrier. However, it is unlikely that all of the monocarboxylic acids tested would display competitive inhibition if lactate was not transported by a carrier. The best evidence is based on the observation that only cells grown in the presence of lactic acid took up the radioactive substrate. An interesting observation concerning the kinetics of the initial rate of uptake of lactic acid is that in F. oxysporum only saturable kinetics were observed for concentrations up to 20 mM. In glucose-grown yeast cells pH-dependent nonsaturable uptake has been observed (9). When we studied the transport of glycerol in this organism, we observed the same type of behavior described above for lactic acid (4). These observations support the hypothesis that peculiarities in the lipid structure of F. oxysporum are responsible for the tight homeostasis of these compounds.

The experimental evidence that the anion lactate was the chemical species that was actually transported under conditions where no H\(^+\) movements were detected raises the problem of how the net charge transported is neutralized under steady-state conditions. A similar situation was described for K. marxianus (6), and in this case the authors postulated that there was a mechanism of antiport with some anion. Bicarbonate might be a good candidate for an antiport with lactate.

From the results described above we concluded that in F. oxysporum lactic acid is transported by a transport mechanism that is common for monocarboxylic acids and is inducible and completely repressed by glucose. Similar mechanisms have been described previously for S. cerevisiae (9) and C. utilis (3).

The diauxic pattern observed during growth of the fungus in liquid medium containing lactic acid and glucose is consistent with the regulation of transport and metabolism of lactic acid described above. The lactate:cytochrome c oxidoreductase (\(2\) or \(4\)) activity was consistently present at high levels in lactate-adapted cells and was absent in glucose- or ethanol-grown cells. These enzymes have been the subjects of extensive investigation since they allow yeasts to grow on both isomers of lactate. In this work we found that F. oxysporum forms a lactate:NAD oxidoreductase. The physiological role of this enzyme may be to yield lactate as an end product of metabolism.

The dramatic decrease in lactate transport activity observed during formation of conidia was also observed for glucose and amino acid transport (data not shown), indicating that decreases in activities of transporters during conidium formation may be a general phenomenon. Certainly regulation phenomena (in particular, regulation of transport during formation of conidia) deserve further research.

**ACKNOWLEDGMENT**

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