# Report Membrane Transport of Lactic Acid in the Filamentous Fungus *Rhizopus*

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## Abstract

The fungus *Rhizopus* is frequently used for fermentative production of lactic acid, but little is known about the mechanisms or proteins for transporting this carboxylic acid. Since transport of the lactate anion across the plasma membrane is critical to prevent acidification of the cytoplasm, we evaluated the functionality of two lactate-proton symport paralogs, LacA and LacB, from *R. delemar*. Both of these proteins showed significant ancestral homology to bacterial lactate permease with 46-50% identity to similar homologs from the genus *Burkholderia*. Based on qPCR, the highest level of expression in *Rhizopus* for the *lacA* gene was on complex medium containing pyruvate, while *lacB* transcript was barely detected with all of the tested culture conditions. A *Saccharomyces cerevisiae jen1* deletion strain lacking the ability to transport monocarboxylates was restored for growth on lactate and pyruvate with the expression of LacA. Expression of the LacB in this same strain did not confer the ability to grow on either carbon source. LacA expression also allowed active transport of L-[$^{14}$C(U)]-lactate into yeast cells and this accumulation was inhibited by the proton uncoupler carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone. Translation fusions with GFP showed that LacA accumulates primarily in the plasma and vacuolar membrane, while LacB is dispersed throughout the cytoplasm. These results indicate that the *Rhizopus* LacA is a functional lactate symport that is probably involved in uptake of pyruvate or lactate, while the physiological role of LacB is unknown.

**Keywords:** *Rhizopus*, lactic acid, metabolic engineering

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# Introduction

Lactic acid (or 2-hydroxypropionic acid) has long been utilized by the food industry as an additive for preservation, flavor, and acidity. More recent expanding markets driven by ecological concerns and desire for petroleum alternatives include the manufacture of the biodegradable plastic, poly-lactic acid (PLA), and the chlorine-free solvent, ethyl lactate (Datta and Henry, 2006). Demand for lactic acid is primarily supplied by microbial fermentation with either the bacteria *Lactobacillus* or the fungus *Rhizopus* (Zhang et al., 2007). Considerable efforts are being invested in enhancing the productivity of current microbial systems and the development of novel bacterial or yeast strains containing genes that confer the ability to synthesize lactic acid. An integral part of this metabolic process involves the export of the carboxylic acid, yet this transport appears to be the least understood step of the fermentative scheme.

Carboxylic acids are transferred across cytoplasmic membranes for both export and import, either by diffusion or by active transport. Since most organisms have a cytosolic pH between 6 to 8, intracellular lactic acid with a pKa of 3.8 is predominantly in the dissociated state. The lactate anion is unable to readily diffuse out of the cell and will accumulate to toxic or inhibitory levels if cellular mechanisms are not employed to remove the acid through oxidative utilization (e.g. metabolic conversion of lactic acid) or by specific transport proteins. Active transport systems may include: primary transporters, which use the energy released from hydrolysis of ATP to pump the solute across the membrane (e.g. ATP binding cassette transporters); or secondary transporters, which rely on the chemiosmotic energy generated from primary transporters to facilitate the movement of molecules across the membrane. Most lactate transporters are considered symports because the lactate anion is transported in the same direction with one or more protons. These lactate-proton symporters usually are indifferent to the isomeric form of the substrate and are often capable of also transporting other short chain monocarboxylates, such as pyruvate and propionate. Lactic acid transporters have been characterized in bacteria, protists, protozoans, yeasts, plants, and animal cells, but not for any filamentous fungi (Casal et al., 2008).

We recently identified two putative lactate symport paralogs, RO3G 07565.3 and RO3G 04839.3 (herein, respectively referred to as LacA and LacB), with similarity to bacterial lactate permease, in the *Rhizopus* genome sequence (www.broad.mit.edu). While this sequenced strain is frequently referred to as *R. oryzae*, it is more accurately classified as *R. delemar* according to proposed delineation that now includes both species. These closely related fungi appear to have nearly identical genetic and phenotypic traits, but differ mainly by the fermentative route used for regeneration of NAD+ necessary for glycolysis. When cells are grown in the presence of fermentative carbon sources, *R. oryzae* synthesizes primarily lactic acid and ethanol, while *R. delemar* produces ethanol, fumaric, and malic acids. *R. delemar* typically does not produce lactic acid and we have not detected it in fermentation studies using the sequenced isolate, *R. delemar* 99-880. Thus, we would not presume that this strain was capable of efficiently transporting lactate; however, we have introduced the lactate dehydrogenase gene, *ldhA*, from *R. oryzae* into *R. delemar*, conferring the ability to synthesize considerable amounts of lactic acid (Skory and Ibrahim, 2007). Thus, we hypothesized that *R. delemar* and *R. oryzae* probably shared a similar lactate transport mechanism. In this work, the genes for the

identified permease, LacA and LacB, were cloned from *R. delemar.* We describe the functionality of these lactate symports and consider their involvement in the transport of lactic acid.

## Materials and Methods

Examination of the *lacA* and *lacB* transcript was initially performed with a cDNA library that we prepared as part of the *Rhizopus* genome sequencing project (Ma et al., 2009). PCR amplifications used the cDNA library as template and primers designed to amplify overlapping regions of each gene in order to confirm the intron splicing pattern. We analyzed the expression of the *lacA* and *lacB* genes in *R. delemar* 99-880 by isolating and testing RNA isolated from cultures grown in YP and RZ (Skory and Ibrahim, 2007) supplemented with 5 g/L of glucose, xylose, glycerol, ethanol, lactate, acetate, or pyruvate. Additionally, isolated *R. delemar* (pLdhA-pyrF) (Skory and Ibrahim, 2007) was tested in RZ-glucose medium with shaking, since we wanted to examine mRNA expression with lactic acid production. We performed real-time quantitative PCR (qPCR) for *lacA* expression using the same cDNA made from this RNA. Detection of *lacA* transcript accumulation used primers QLacA-1 and QLacA-2 for amplification of a 173-bp product with a 5' nuclease probe, QLacA-Probe, labeled with 5' 6FAM and 3' BHQ-I (Integrated DNA Technologies, Coralville, IA). Samples were normalized to the 18s rRNA quantity determined with primers Q18S-1 and Q18S-2 for amplification of a 83-bp product and Q18S-Probe also labeled with 5' 6FAM and 3' BHQ-I. All samples, standards, and controls were performed in triplicate.

The coding regions of *lacA* and *lacB* were cloned into pRH160 containing the *PGK1* promoter ($P_{PGK}$). The resulting plasmids were then referred to as pLacA-160 and pLacB-160, respectively. Plasmid pRH160 was constructed in this study by PCR amplification of the $P_{PGK}$ and *PGK* terminator ($T_{PGK}$) from *S. cerevisiae* BY4727 genomic DNA with primers Pgk1P-SacI/Pgk1P-SpeI and Pgk1T-SalI/Pgk1T-XhoI, respectively. Sequence-verified promoter and terminator fragments were then sub-cloned into the yeast shuttle vector pRS426 (Christianson et al., 1992) using the corresponding *Sac*I/*Spe*I and *Sal*I/*Xho*I restriction endonuclease sites. Constructs were also created by overlapping PCR amplification with fusions of the S65T variant (Heim et al., 1995) of green fluorescent protein (GFP) at the carboxyl termini of the *lacA* and *lacB* genes.

Transport assays were performed in a manner similar to those described by Makuc *et al.* (Makuc et al., 2001). We first grew *S. cerevisiae* JMY75 transformed with pRH160, pLacA-160, and pLacB-160 in SM medium supplemented with 2% ethanol and 10 mM alanine. Cells were harvested during exponential growth, washed in ice-cold water, re-suspended to a final concentration between 30-60 mg (dry weight) per ml water, and then kept on ice. Reactions were prepared by mixing 30 µl of cells with 30 µl 0.2 M potassium phosphate buffer (pH 6.0), pre-incubating 2 min at 25˚C, and then initiating the reactions with the addition of 20 µl of 4x pre-warmed sodium L-lactate at the desired concentration. The 4x lactate contained sodium L-[$^{1}$4C(U)]-lactate (PerkinElmer, Boston, MA) at a constant radioactivity of 5E-4 µCi/nmol of total lactate. After the desired period of time, between 10 sec to 4 min, of incubation at 25˚C, reactions were stopped by the dilution with 5 ml ice-cold water and immediate filtering of cells through GF/C membranes (Whatman, Clifton, NJ), followed with an additional 10 ml of ice-cold water rinse. For analysis of non-specific L-[$^{14}$C(U)]-lactate absorption, labeled lactate was added

after the initial 5 ml of cold water, followed immediately by filtration and rinsing. We also tested the effects of carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) by performing reactions in the presence of this proton uncoupler at a concentration of 100 μM. All assays were performed in triplicate for each condition. Kinetic analyses were calculated with the Enzyme Kinetic Module for SigmaPlot (Systat Software, San Jose, CA) using non-linear fit of the Michaelis-Menten Equation.

## Results and Discussion

The objectives of this study were to determine if LacA and LacB from *Rhizopus* were functional lactate symports and to ascertain whether they were involved in lactate transport in *Rhizopus*. Sequencing of fragments amplified from our *R. delemar* 99-880 cDNA library, RACE analysis, and comparisons to closely related proteins revealed that each lactate permease gene contained eight exons encoding putative proteins 595 and 568 amino acids for the LacA and LacB, respectively. All of the *lacA* cDNA isolates from the RACE analysis had transcriptional start sites of -16nt, while the *lacB* isolates had multiple start sites. Phylogenetic comparisons of the *Rhizopus* LacA and LacB proteins to closely related homologs clearly demonstrate the similarity to bacterial lactate permease. The lactate permease proteins are subdivided into distinct clades consisting of Bacilli and beta, gamma, and alpha proteobacteria. The greatest homology of the fungal lactate permease appears to be with the beta-proteobacteria, or more specifically to the genus *Burkholderia*. Homology with this genus ranged from 46 to 50% within the coding region. The LacA and LacB did not have any significant homology to any confirmed yeast lactate transporters in *S. cerevisiae* (Casal et al., 1999) and *Kluveromyces lactis* (Queiros et al., 2007).

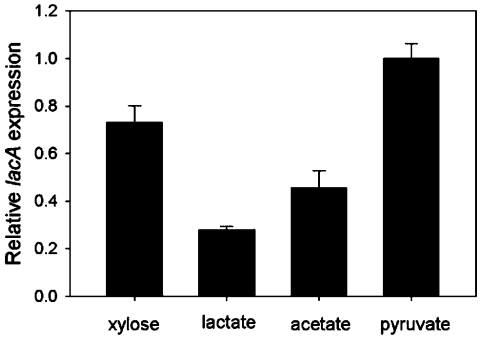


**Figure 1.** Relative transcript accumulation of *lacA* in *R. delemar* 99-880

We initially tried to quantify the *lacA* and *lacB* RNA from *R. delemar* with northern hybridization analysis, but lactate permease transcript levels were too low to detect with any of the culture conditions tested in this study. Therefore, we relied on qPCR with cDNA made from this same RNA. The highest levels of *lacA* accumulation were found with cDNA samples made from RNA isolated from submerged cultures grown for 24 h in YP with pyruvate, followed by xylose, acetate, and lactate, suggesting that this gene may play a role in uptake of pyruvate or lactate (Figure 1.). Likewise, all of the cultures grown on minimal medium, including the recombinant *R. delemar* (pLdhA-PyrF) grown with conditions known to produce lactic acid, were all too low with *lacA* amplification to be of any significant value. We did not attempt qPCR analysis of the *lacB* gene, because of the lack of reproducible amplification with the growth conditions tested in this study.

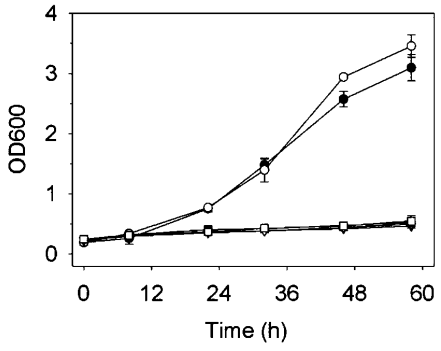


**Figure 2.** Growth of *S. cerevisiae* JMY75 transformants with lactate or pyruvate. Transformants containing pRH160 (squares), pLacA-160 (circles), or pLacB-160 (triangles) were grown in SM with 2% ethanol and either 20 mM pyruvate (open symbols) or 20 mM DL-lactate (closed symbols).

We tested the functionality of lactate permease genes in the $P_{PGK}$ expression plasmid pRH160 in *S. cerevisiae* JMY75. This particular strain is unable to transport lactate and pyruvate due the lactate symport gene, *JEN1*, being disrupted. It is able to utilize ethanol as the main carbon source; however, deletions in the pyruvate kinase and malate dehydrogenase genes prevent the synthesis of pyruvate which is required for synthesis of some amino acids like alanine, leucine, isoleucine, and valine (Makuc et al., 2004). Therefore, growth on ethanol and either pyruvate or lactate requires a functional transport mechanism to allow growth with these monocarboxylates. *S. cerevisiae* JMY75 (pLacA-160) grown in SM with 2% ethanol and either 20 mM pyruvate or 20 mM DL-lactate clearly demonstrate the ability of *S. cerevisiae* to transport either of these carboxylic acids (Figure. 2). Significantly equivalent growth densities of over $OD_{600nm}$=3 were achieved with either pyruvate or lactate, while little or no growth occurred for pLacB-160 or the vector pRH160.

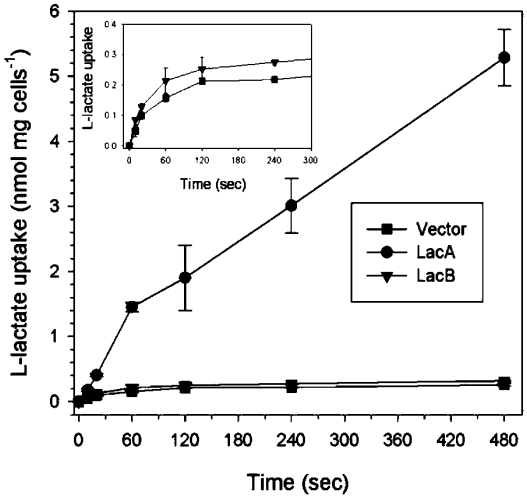


**Figure 3.** Time course analysis of L-[$^{14}$C(U)]-lactate uptake by *S. cerevisiae* JMY75 transformants. Isolates containing pLacA-160 (circle), pLacB-160 (triangle), or vector control pRH160 (square) were examined for linearity of substrate uptake over a period of 8 min. Inset contains data for only pLacB-160 and pRH160 to better display the curve.

A time course study of L-[$^{14}$C(U)]-lactate to determine the period of zero order kinetics for the initial uptake rates showed that all of the *S. cerevisiae* transformants appeared to have linearity of L-[$^{14}$C(U)]-lactate uptake for up to 40-60 sec after the addition of labeled substrate. *S. cerevisiae* (pLacA-160) continued to take up L-[$^{14}$C(U)]-lactate with no apparent saturation for up to eight minutes, indicating active transport of the substrate (Figure. 3). In comparison, *S. cerevisiae* transformed with either pLacB-160 or the vector control pRH160 quickly started to plateau for substrate uptake within 60 sec and never got above 0.3 nmol L-[$^{14}$C(U)]-lactate mg cells$^{-1}$ during the 8 min. The kinetics of L-[$^{14}$C(U)]-lactate uptake was only tested for *S. cerevisiae* (pLacA-160), since pLacB-160 and the vector control pRH160 appeared to be non-functional

and only capable of passive uptake of the substrate. The $K_m$ and $V_{max}$ for *S. cerevisiae* (pLacA-160) were calculated by Michaelis-Menten equation to be 0.89 (±0.18) mM L-lactate and 0.33 (±0.02) nmol L-lactate/ mg dry cell/sec, respectively, with an $R^2$ of 94%. Adding the proton motive force inhibitor FCCP to reactions containing 0.5 mM and 4 mM L-lactate resulted in a 79% ($p= 0.005$) and 64% ($p=0.001$) decrease in substrate uptake, respectively, when compared to the non-treated controls. This strongly suggests that this permease is driven by a proton motive force and is likely a lactate-proton symport

We believe that the inability to transport monocarboxylates in *S. cerevisiae* expressing LacB is likely related to the inability of this protein to localize to the plasma membrane. Fusions with GFP suggest that only the LacA-GFP is targeted to the plasma membrane, while the majority of the LacB-GFP was dispersed throughout the cytoplasm. This may be the typical localization of LacB or the GFP fused to LacB may cause improper translocation. Isolates expressing the LacA-GFP also seemed to have a considerable amount of fluorescence associated with what appears to be the vacuolar membrane, which could be the result of excess misfolded proteins being targeted for degradation or the normal endocytic activity of plasma membrane recycling to the vacuole (Huang and Klionsky, 2002). The difficulties with detecting *lacB* transcript and the inability to show functional transport in *S. cerevisiae* suggests that this enzyme does not have a physiological role with lactic acid utilization or export. Therefore, we are currently in the process of examining other putative lactate transporters in *Rhizopus* to better understand the mechanism of lactate export in this fungus.

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