Transport of Glucose and Cellobiose by Candida wickerhamii and Clavispora lusitaniae*

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The cellular location of $\beta$-1,4-glucosidase activity from, as well as the transport of glucose and cellobiose into, cells of Clavispora lusitaniae NRRL Y-5394 and Candida wickerhamii NRRL Y-2563 was investigated. The $\beta$-glucosidase from Cl. lusitaniae appeared to be a soluble cytoplasmic enzyme. This yeast transported both glucose and cellobiose when grown in medium containing cellobiose as the sole carbon source. Glucose, but not cellobiose, uptake was observed for cells grown on glucose. The $K_s$ and $V_{max}$ values for cellobiose transport were different when Cl. lusitaniae was cultured either aerobically (0.11 mM, 6.28 nmol·min$^{-1}$·mg$^{-1}$) or anaerobically (0.25 mM, 3.88 nmol·min$^{-1}$·mg$^{-1}$). The $K_s$ and $V_{max}$ values for glucose transport (0.23–1.10 mM and 17.2–33.9 nmol·min$^{-1}$·mg$^{-1}$) also differed with the various growth conditions. The $\beta$-glucosidase from C. wickerhamii was extracytoplasmically located. This yeast transported glucose, but not cellobiose, under all growth conditions tested. The $K_s$ for glucose uptake was 0.13–0.28 mM when C. wickerhamii was cultured on cellobiose and 0.25–0.30 mM when cultured on glucose. The $V_{max}$ values for glucose uptake were greater for cells cultured on cellobiose (35.0–37.9 nmol·min$^{-1}$·mg$^{-1}$) than for cells cultured on glucose (15.6–21.4 nmol·min$^{-1}$·mg$^{-1}$). Cellobiose did not inhibit glucose uptake in either yeast. Glucose partially inhibited cellobiose transport in C. lusitaniae, but only if the yeast was grown aerobically. In both yeasts, sugar transport was sensitive to carbonyl cyanide p-trifluoromethoxyphenylhydrazone and 1799, but insensitive to valinomycin.

Fermentation of cellobiose to ethanol is a relatively rare fungal trait. Of the 308 species of yeasts that are known to assimilate cellobiose as a sole carbon source, only 12 species ferment it to ethanol (1). A screen performed in this laboratory demonstrated that Clavispora lusitaniae NRRL Y-5394 and Candida wickerhamii NRRL Y-2563 had faster initial rates of ethanol production from cellobiose than did the other cellobiose-fermenting yeasts tested (2). Although both yeasts are capable of rapidly fermenting cellobiose, previous observations indicated that different metabolic mechanisms might be employed. For instance, substrate utilization studies showed that Cl. lusitaniae could ferment only glucose and cellobiose, while C. wickerhamii fermented glucose, cellobiose, and celodextrins of polymerization degree three through six (3). The ability of C. wickerhamii to ferment longer chain celodextrins is apparently due to the presence of an extracellular $\beta$-glucosidase (EC 3.2.1.21) that hydrolyzes celodextrins to glucose (4–6). This enzyme has been purified and partially characterized (5–7), and the physiological factors that regulate its expression have been investigated. The C. wickerhamii $\beta$-glucosidase is produced constitutively; however, when grown aerobically, high concentrations of glucose repress its expression. Surprisingly, anaerobiosis appears to overcome the glucose repression (4). From the enzyme characterization and substrate utilization studies, it was postulated (2, 5, 6) that C. wickerhamii might not transport cellobiose across its cytoplasmic membrane, but rather, first hydrolyze cellobiose and then transport the resultant glucose. In contrast, Cl. lusitaniae does not appear to produce an extracellular $\beta$-glucosidase (2). Thus, it has been postulated that this yeast might first transport cellobiose across its cytoplasmic membrane and then hydrolyze it (2, 3). To date, no direct experimental evidence to test these hypotheses has been reported, although the glucose transport systems of C. wickerhamii have recently been examined in some detail (8–10).

This report characterizes the cellular location of the $\beta$-glucosidase activity in Cl. lusitaniae and C. wickerhamii and investigates the transport of radiolabeled glucose and cellobiose into intact cells of the two yeasts grown under various physiological conditions. To our knowledge, this is the first report presenting direct evidence that a yeast transports cellobiose across its cytoplasmic membrane and, furthermore, characterizes some of the properties of a cellobiose carrier. A model is also presented that depicts the different mechanisms by which these yeasts utilize glucose and cellobiose.

**MATERIALS AND METHODS**

*Sources of Chemicals, Organisms, and Inocula—Yeast extract, malt extract, peptone, glucose, agar, and yeast nitrogen base medium were purchased from Difco Laboratories, Detroit, MI. Cellobiose, $\alpha$-L-phosphoglucone, glucose 6-phosphate, p-nitrophenyl $\beta$-D-glucopyranoside (pNPG), valinomycin, carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), hexokinase, and adenosine triphosphate were purchased from Sigma. [U-$^{14}$C]Glucose (10.31 Bq/mmol) was purchased from Du Pont-New England Nuclear Research Products. All other chemicals were reagent grade. Prior to use, cellobiose was recrystallized from ethanol to remove trace impurities of glucose. [14C]Cellobiose was synthesized as previously described by utilizing crude enzyme preparations from Cellulibrio gilus (11). The synthesized cellobiose was purified by charcoal-Celite chromatography (12). NRRL B-14074 Cu. gilus Hutchler & King, NRRL Y-2563 C. wickerhamii (Caprotti) Meyer & Yarrow (syn. Tolypopsis wickerhamii),

The abbreviations used are: pNPG, p-nitrophenyl $\beta$-D-glucopyranoside; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

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and NRRL Y-5394 *C. lusitaniae* Rodrigues de Miranda (syn. *Candida lusitaniae*) were obtained from the Agriculture Research Service Culture Collection, Northern Regional Research Center, Peoria, IL.

Inocula preparation and incubation conditions were previously described (2). Twenty milliliters of basal YM medium (0.3% yeast extract, 0.3% malt extract, 0.5% peptone) containing 20 g of glucose/liter was inoculated with a loopful of yeast. After 24 h, 0.1 ml of culture was used to inoculate 20 ml of fresh YM medium containing 20 g of glucose/liter. The cells were incubated for 24 h, harvested by centrifugation at 8000 × g for 10 min, washed twice in sterile distilled water, and used to inoculate (1.5% v/v) the experimental flasks. Flasks containing 25 ml of medium were capped with either cotton stoppers (for aerobic growth) or serum stoppers and vented with No. 25-gauge needles (for anaerobic growth). All incubations were performed on a rotary shaker (250 rpm) at 28 °C.

**Trapping Experiment**—Preliminary experiments2 showed that 0.67\% Difco yeast nitrogen base medium supplemented with 0.25% sodium succinate (pH 6.0) and 0.02% yeast extract was the optimal basal synthetic medium for \( \beta \)-glucosidase production and cellobiose fermentation with *C. wickerhamii*. However, minimal growth occurred in this medium, even in the presence of nonfermentable substrates. Therefore, carbon utilization of fermentable substrates was monitored by ethanol formation.

The "trapping" experiment was performed with cells grown anaerobically in the basal synthetic medium initially supplemented with 0.02 M MgCl\(_2\), 0.07 M ATP, 24 units/ml hexokinase, and 25 g of either cellobiose or glucose 6-phosphate per liter. Additional ATP (0.375 mmol) and hexokinase (150 units) were added to the flasks after 1 and 2 days of incubation. Samples were removed at various times after inoculation, and the amount of ethanol was determined by GLC as previously described (2).

**Measurement of \( \beta \)-Glucosidase Activity and Cellular Location**—The *C. wickerhamii* and *C. lusitaniae* \( \beta \)-glucosidase assays were performed in 0.01 M sodium acetate (pH 4.75) (5) or 0.01 M sodium phosphate (pH 6.5) buffer, respectively. These correspond to the pH optima of the respective enzymes. Appropriately diluted samples were mixed with 0.5 ml of buffered 3.5 mM pNPG and incubated at 28 °C for 15 min. The reactions were stopped by the addition of 0.5 ml of 1 M glycine (pH 10.8), and the amount of p-nitrophenol liberated was quantitated spectrophotometrically at 400 nm (2). One unit of activity is defined as the amount of enzyme that produces 1 \( \mu \)mol of p-nitrophenol per min under the above prescribed conditions.

To determine the cellular location of the enzymes, the yeasts were grown for 1 day in YM medium containing 10 g of cellobiose per liter. Assays were initially performed on the cells plus medium. The cells were harvested by centrifugation at 8000 × g for 10 min, resuspended in the original volume of buffer, and the cells and medium were assayed. Cells were broken by vortexing at maximum speed for 4 min after the addition of 0.25-0.5 mm glass beads (13). The cell suspensions were homogenized at 10,000 × g for 10 min, and the pellets were resuspended in buffer. The 10,000 × g supernatant was then centrifuged at 100,000 × g for 1 h. Assays were performed on the supernatants and the resuspended pellets. Spheroplasts of *C. wickerhamii* were prepared with Zymolyase 60,000 (Miles Laboratories, Elkhart, IN) as previously described (5). The spheroplasts were harvested by centrifugation at 3000 × g for 5 min, lysed by resuspension in 0.01 M sodium acetate (pH 4.75), and further fractionated by centrifugation at 10,000 × g for 10 min (Table 1).

**Measurement of Glucose and Cellobiose Uptake Rates**—Uptake experiments were conducted with cells grown either aerobically or anaerobically in YM medium containing either glucose (100 g/liter), cellobiose (100 g/liter), or sodium succinate (50 g/liter) (pH 6.0). After a 24-h incubation, 25 ml of cells were harvested by centrifugation at 8000 × g for 10 min, washed twice in sterile distilled water, and resuspended in 2.5 ml of sterile transport buffer composed of 0.01 M Na\(_2\)HPO\(_4\), 0.01 M KH\(_2\)PO\(_4\), 0.2 M KCl, 0.01 M MgSO\(_4\), and either 0.001 M CaCl\(_2\) (pH 6.6, *C. lusitaniae*) or 0.001 M CaCl\(_2\) (pH 4.75, *C. wickerhamii*). Prior to use, the cells were starved for 4 h with shaking at 28 °C. Similar results were obtained if the cells were starved from 2 to 24 h. From this cell suspension, 10- to 20-μl aliquots were mixed with various amounts of transport buffer to a final volume of 0.1 ml. The reactions were initiated by the addition of either D-[U-\(^1\)C]glucose or \(^1\)C\)cellobiose. After a 24-h incubation at 25 °C, the reactions were terminated by addition of 3 ml of cold transport buffer, and the reactions were placed on ice. The suspensions were filtered through Millipore HA WP membranes (0.45 μm), washed with 3 × 3 ml of transport buffer, and counted in 10 ml of ACS II scintillation fluid (Amersham). Preliminary experiments were performed to ensure that uptake was linear with time and cell concentration.

**RESULTS**

To examine the general mechanisms by which *C. lusitaniae* and *C. wickerhamii* metabolized cellobiose, a classical trapping experiment (14) was performed in synthetic medium with cellobiose as the fermentable substrate. Most species of yeast are unable to transport glucose 6-phosphate. Therefore, if a yeast extracellularly cleaves cellobiose and then transports glucose, a significant lag in the metabolism of cellobiose should be observed upon addition of hexokinase (EC 2.7.1.1) and ATP to the medium. If, however, the yeast transports cellobiose across its cytoplasmic membrane and then hydrolysis occurs, no effect of the added hexokinase or ATP should be observed. The results of such an experiment are presented in Fig. 1. Cellobiose metabolism was monitored via the production of ethanol. *C. wickerhamii* displayed a significant lag in ethanol production (approximately 24–36 h), as compared to the control culture, when hexokinase and ATP were added to the medium, suggesting that this yeast transported predominantly glucose (Fig. 1A). In contrast, no lag in ethanol production was observed in the *C. lusitaniae* cultures, indicating that they transported cellobiose (Fig. 1B). As expected, no ethanol production was observed for either yeast when glucose 6-phosphate was substituted for cellobiose.

The subcellular locations of the \( \beta \)-glucosidase activity in *C. lusitaniae* and *C. wickerhamii* are characterized by the data presented in Table 1. The *C. lusitaniae* \( \beta \)-glucosidase was found to be a soluble, cytoplasmically located enzyme. No activity was detected with whole cells or in the culture supernatant; however, activity was readily detected in broken cells. Most of this activity (93%) remained soluble after the cell debris was removed by centrifugation. The enzyme also did not appear to be membrane-bound as 97% of the activity remained in the supernatant after centrifugation at 100,000 × g for 1 h. The *C. wickerhamii* enzyme was located extracytoplasmically yet cell-associated, with only a small amount (<20%) located in the cytoplasm. \( \beta \)-Glucosidase activity was readily detected in the cells plus medium, but only a minor fraction of the activity (<15%) was present in the cell-free medium. Upon breakage of the cells, a small increase in total

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2 S. N. Freer, unpublished results.

3 S. N. Freer, unpublished experiments.
activity (20%) was observed. About one-half of the activity pelleted upon centrifugation at 10,000 × g for 10 min, indicating that the enzyme was cell-bound. Almost all of the β-glucosidase activity was solubilized upon treatment by Zymolyase 60,000, which hydrolyzes linear glucose polymers with β-1,3-linkages and results in the formation of spheroplasts. Upon lysis of the isolated spheroplasts, only a minor increase (≈15%) in the total activity was detected.

The results of the trapping experiment and the cytoplasmic location of the Cl. lusitaniae β-glucosidase activity suggested that the yeast transports cellobiose across its cytoplasmic membrane. Cellobiose and glucose uptake experiments were performed with Cl. lusitaniae whole cells that were cultured either aerobically or anaerobically on both substrates. Uptake followed Michaelis-Menten kinetics, and estimates for the half-saturation constant ($K_s$) and capacity ($V_{max}$) were obtained from linear regression analysis of Lineweaver-Burk plots (Table II). Cl. lusitaniae cultured on cellobiose indeed transported the disaccharide. However, no cellobiose uptake was observed for cells that were cultured in medium containing glucose. The cellobiose carrier appeared to be glucose-repressed because Cl. lusitaniae cultured aerobically in medium containing either succinate or xylose as the sole carbon source exhibited cellobiose uptake. Cellobiose transport appeared to be influenced by oxygen availability. When cells were grown aerobically, the $K_s$ for cellobiose uptake was 0.11 mM, and the $V_{max}$ was 3.88 nmol·min⁻¹·mg⁻¹. The $K_s$ was 0.25 mM, and the $V_{max}$ was 6.28 nmol·min⁻¹·mg⁻¹ for anaerobically grown cells. Oxygen availability also appeared to influence glucose transport in Cl. lusitaniae. This was more apparent for cells cultured on cellobiose, which had $K_s$ values for glucose uptake of 1.10 mM and 0.23 mM, respectively, for cells grown aerobically or anaerobically. The $K_s$ values for glucose-grown cells were 0.84 mM and 0.52 mM under aerobic and anaerobic conditions, respectively. $V_{max}$ values ranged from 17.2 to 33.9 nmol·min⁻¹·mg⁻¹ for the various growth conditions.

### Table I

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cl. lusitaniae</th>
<th>C. wickerhamii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells and medium</td>
<td>0</td>
<td>4.2</td>
</tr>
<tr>
<td>Medium</td>
<td>0</td>
<td>0.7</td>
</tr>
<tr>
<td>Cells</td>
<td>0</td>
<td>4.1</td>
</tr>
<tr>
<td>Broken cells</td>
<td>9.4</td>
<td>5.2</td>
</tr>
<tr>
<td>centrifuged at 10K</td>
<td>8.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Supernatant</td>
<td>0.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Pellet</td>
<td>6.5</td>
<td>2.4</td>
</tr>
<tr>
<td>Supernatant at 10K</td>
<td>0.2</td>
<td>0.2</td>
</tr>
</tbody>
</table>

| Spheroplasts       | —             | 3.7           |
| Centrifuged at 10K | —             | 3.6           |
| Supernatant        | —             | 0.2           |
| Pellet             | —             | 0.6           |

* The substrate was 2.5 mM pNPG.
* The assays were performed in 0.01 M NaOAc (pH 6.5).
* The assays were performed in 0.01 M NaOAc (pH 4.75).
* —, assay not conducted.

### Table II

**Kinetic parameters of cellobiose and glucose uptake in Cl. lusitaniae**

Data reflect average values ± S.E. determined from three independent cultures.

<table>
<thead>
<tr>
<th>Carbon source</th>
<th>Cellobiose uptake</th>
<th>Glucose uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_s$ (mM)</td>
<td>$V_{max}$ (nmol·min⁻¹·mg⁻¹)</td>
</tr>
<tr>
<td>Glucose + O₂</td>
<td>0.11 ± 0.01</td>
<td>3.88 ± 0.45</td>
</tr>
<tr>
<td>Glucose - O₂</td>
<td>0.25 ± 0.01</td>
<td>6.28 ± 0.82</td>
</tr>
</tbody>
</table>

* $G_i$ represents glucose and $G_z$ represents cellobiose.
* —, no uptake was detected.

![Fig. 2. Inhibition of labeled glucose uptake by glucose (●) or cellobiose (▲) in Cl. lusitaniae grown either anaerobically (A) or aerobically (B) in medium containing either glucose (A) or cellobiose (B) as the carbon source.](image1)

![Fig. 3. Inhibition of labeled cellobiose uptake by glucose (●) or cellobiose (▲) in Cl. lusitaniae grown either anaerobically (A) or aerobically (B) in medium containing cellobiose as the carbon source.](image2)
An analogous set of transport experiments were conducted with *C. wickerhamii*, and the results are presented in Table III. No measurable cellobiose uptake was observed under any growth condition examined. Significant glucose uptake was always observed, and, as the *V*<sub>max</sub> values indicated, uptake was greater in cellobiose-grown cells. For cellobiose-grown cells, the *K*<sub>m</sub> values were somewhat different under aerobic and anaerobic conditions (0.29 mM and 0.18 mM, respectively). However, the standard error of these assays makes it difficult to assess the physiological significance of these data. For glucose-grown cells, oxygen availability did not affect the kinetic parameters of glucose uptake.

Figs. 2-4 present selected examples that examine the cross-inhibition by glucose and cellobiose on their uptake in the two yeast species. Glucose uptake in *C. lusitaniae* cultured aerobically on either glucose (Fig. 2A) or cellobiose (Fig. 2B) was either not inhibited or only slightly inhibited by cellobiose. This was also the result observed if *C. lusitaniae* was cultured anaerobically on these substrates. Similarly, glucose did not inhibit cellobiose transport when the cells were grown anaerobically on cellobiose (Fig. 3A). However, partial glucose inhibition of cellobiose transport was observed if *C. lusitaniae* was grown aerobically on cellobiose (Fig. 3B). In *C. wickerhamii* (which only transports glucose, see Table III) no inhibition of glucose transport by cellobiose was observed, regardless if the cells were cultured anaerobically on glucose (Fig. 4A) or cellobiose (Fig. 4B). Similar results were obtained if the cells were cultured aerobically. As expected, inhibition of labeled substrate transport by cold substrate approached saturation for all experiments.

**Table IV**

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>Glucose transport</th>
<th>Cellbiose transport</th>
<th>Glucose transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>C. lusitaniae</em></td>
<td><em>C. wickerhamii</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Val</td>
<td>FCCP</td>
<td>1799</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>103</td>
<td>40</td>
<td>62</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>110</td>
<td>38</td>
<td>64</td>
</tr>
<tr>
<td>Cellbiose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aerobic</td>
<td>90</td>
<td>37</td>
<td>61</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>97</td>
<td>26</td>
<td>53</td>
</tr>
</tbody>
</table>

*Cells grown on glucose do not transport cellbiose.*

Aside from group translocation, where the substrate crosses the membrane and at the same time it is chemically altered, transport mechanisms can be placed in two general categories: facilitated diffusion, in which the substrate passively crosses the membrane mediated by a carrier, and active transport, in which the substrate and an ion cross the membrane in a coupled reaction. Transport coupled to H<sup>+</sup> is most commonly reported in mammalian systems (15), while the coupling ion is generally H<sup>+</sup> in bacteria (16). Although few systems have been investigated, fungi also appear to couple active transport to H<sup>+</sup> (17–19). Assuming that in *C. lusitaniae* and *C. wickerhamii* sugars and a cation are actively translocated together, transport should be inhibited by ionophores. The data presented in Table IV show that valinomycin did not inhibit glucose transport in either yeast. Likewise, valinomycin did not inhibit cellobiose uptake in *C. lusitaniae*. In contrast, FCCP dramatically inhibited sugar uptake in both yeasts, suggesting that transport is directly coupled to H<sup>+</sup>. This possibility is supported by the fact that, in both yeasts, glucose uptake was also inhibited by 1799. However, it remains unexplained as to why 1799 appeared to have little effect on *C. lusitaniae* cellobiose transport.

**DISCUSSION**

Even though *C. wickerhamii* and *C. lusitaniae* both metabolize cellobiose efficiently, they have evolved different mechanisms to do so. As depicted by the model shown in Fig. 5, *C. lusitaniae* transports cellobiose across its cytoplasmic membrane where a cytoplasmic β-glucosidase hydrolyzes the disaccharide to two glucose moieties. From the kinetic data, this yeast appears to produce at least two cellobiose carriers whose expression is influenced by oxygen availability. This hypothesis is supported by the fact that glucose appeared to inhibit cellobiose uptake if the cells were grown aerobically (Fig. 3B), but not if the cells were grown anaerobically (Fig. 3A). The cellobiose carriers appear to be glucose-repressed, since cells grown aerobically on either succinate or xylose also transported cellobiose, while cells grown on glucose did not. Interestingly, β-glucosidase synthesis is also glucose-repressed, suggesting the possibility of coordinate regulation. *C. lusitaniae* also appeared to have multiple glucose transport systems (Table II) that are independent of the cellobiose transporters because cellobiose did not inhibit glucose uptake (Fig. 2). Both the glucose and cellobiose transport systems were sensitive to the protonophore FCCP, suggesting that they might function by proton symport.

Unlike *C. lusitaniae*, *C. wickerhamii* cannot transport cellobiose (Table III). *C. wickerhamii* produces a cell-associated, extracytoplasmic β-glucosidase (Table I, see Fig. 5). This yeast...
must first hydrolyze cellobiose to glucose and subsequently transport it. Our results suggest that C. wickerhamii constitutively produces a single glucose carrier (K<sub>s</sub> = 0.13–0.30 mM, Table III) that is insensitive to the presence of cellobiose (Fig. 4). Spencer-Martins and van Uden (8) reported the presence of two glucose carriers in C. wickerhamii. If the yeast was grown in medium containing glucose, glucose was apparently transported into the cells via facilitated diffusion (K<sub>s</sub> = 1.7 mM), while cells grown in medium containing cellobiose transported glucose via proton symport (K<sub>s</sub> = 0.18 mM) (8). We were able to detect the higher affinity, active transport system; however, we did not detect the presence of a glucose transporter that functioned by facilitated diffusion. Regardless of the growth conditions, glucose uptake was sensitive to the protonophores 1799 and FCCP, suggesting that the glucose transporter we described does not function by facilitated diffusion. We found no inhibition of glucose transport by cellobiose (Fig. 4). Spencer-Martins and van Uden (10) reported that cellobiose did inhibit glucose transport; however, the K<sub>s</sub> was quite high, 80 mM. All of the commercially available cellobiose we have examined to date is contaminated with small amounts of glucose, which might explain the high K<sub>s</sub> previously reported for cellobiose inhibition of glucose transport (10). The conflicting results might also be due to differences in media, growth conditions, and/or strain histories.

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REFERENCES