**Saccharomyces cerevisiae** Engineered for Xylose Metabolism Exhibits a Respiratory Response

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Received 12 May 2004/Accepted 24 June 2004

Native strains of *Saccharomyces cerevisiae* do not assimilate xylose. *S. cerevisiae* engineered for D-xylose utilization through the heterologous expression of genes for aldose reductase (XYL1), xylitol dehydrogenase (XYL2), and D-xylulokinase (XYL3 or AKS1) produce only limited amounts of ethanol in xylose medium. In recombinant *S. cerevisiae* expressing XYL1, XYL2, and XYL3, mRNA transcript levels for glycolytic, fermentative, and pentose phosphate enzymes did not change significantly on glucose or xylose under aeration or oxygen limitation. However, expression of genes encoding the tricarboxylic acid cycle, respiration enzymes (HXX1, ADH2, COX13, ND11, and NDE1), and regulatory proteins (HAP4 and MTH1) increased significantly when cells were cultivated on xylose, and the genes for respiration were even more elevated under oxygen limitation. These results suggest that recombinant *S. cerevisiae* does not recognize xylose as a fermentable carbon source and that respiratory proteins are induced in response to cytosolic redox imbalance; however, lower sugar uptake and growth rates on xylose might also induce transcripts for respiration. A petite respiration-deficient mutant (pρ) of the engineered strain produced more ethanol and accumulated less xylitol from xylose. It formed characteristic colonies on glucose, but it did not grow on xylose. These results are consistent with the higher respiratory activity of recombinant *S. cerevisiae* when growing on xylose and with its inability to grow on xylose under anaerobic conditions.

Xylose is one of the most abundant carbohydrates in nature. As a structural analog of glucose, it forms the backbone for glucuronoxylans—the predominant hemicellulose of angiosperms (44). Many fungi and bacteria will grow on xylose aerobically, but relatively few will produce ethanol from it. Of 689 recognized yeast species, 154 will both ferment glucose and xylose (44). Many fungi and bacteria will grow on xylose glucuronoxylans—the predominant hemicellulose of angiosperms, and trace amounts of ethanol from xylose (53). When NADPH is a cofactor in the first step, the reaction is tied to NADPH production. The second step is coupled to reduction of NAD+, which can create a cofactor imbalance when oxygen or respiration is limiting (4). Naturally occurring yeasts that metabolize xylose anaerobically have an aldose reductase that also accepts NADH (56). Yeasts that ferment D-xylose require oxygen for growth on the sugar (11, 39). No known native eukaryote will grow on xylose anaerobically.

The discovery that yeasts can ferment D-xylose (7, 59) prompted genetic engineering of xylose fermentation in *Saccharomyces cerevisiae*. Heterologous expression of xylose isomerase (1, 36) has had periodic reports of success (54), but most efforts have introduced genes coding for xylose reductase (XYL1), xylitol dehydrogenase (XYL2) and D-xylulokinase (EC2.7.1.17) (XYL3 or AKS1) (25, 29, 30, 32, 33, 51). Recombinant *S. cerevisiae* expressing these three genes for xylose assimilation can grow on xylose as a sole carbon source, but its capacity for ethanol production from xylose depends upon oxygen availability. In this respect, its xylose metabolism is similar to those of native xylose-fermenting yeasts (18). Very recently, uncharacterized mutations in engineered *S. cerevisiae* have been shown to impart the capacity for anaerobic growth on xylose (50). Metabolic regulation by glucose has been studied in *S. cerevisiae* for many years (16). The regulatory and physiological properties of xylose metabolism have been extensively studied only in the xylose-fermenting yeast *Pichia stipitis* (8, 43), which has served as the source of genes for engineering xylose metabolism in *S. cerevisiae*.

It is crucial to understand the regulatory mechanisms of xylose metabolism, especially if we are to engineer a functional pathway in this nonnative xylose-fermenting organism. In the present study, we used DNA microarrays to investigate how transcriptional regulation of *S. cerevisiae* differs for xylose and glucose metabolism, and we confirmed the regulation of critical genes by real-time PCR (RT-PCR). We tested two hypotheses rationalizing the low level of production of ethanol from xylose: either growth on xylose does not induce transcripts for glycolytic and fermentative enzymes, or growth on xylose does not repress respiration. As predicted by transcriptional-profiling studies, xylose-grown cells were predominantly aerobic, and we were able to improve xylose fermentation by blocking respiration.

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**MATERIALS AND METHODS**

Yeast strains and growth conditions. *S. cerevisiae* YSX3 (MATα leu2-3::LEU2-XYL1 ura3-1::URA3-XYL2 Ty3::NEO-XYL3) was grown in YP medium as described previously (30). The cells were grown under full aeration or...
oxygen limitation, with glucose or xylose as a carbon source (four conditions). The cells were cultured with full aeration in 200 ml of YP medium with 20 g of either glucose or xylose/liter in 1,000-ml flasks shaken at 300 rpm and were harvested at an optical density at 600 nm (OD\textsubscript{600}) of 1. For simulation of real fermentative conditions, oxygen-limited cells were cultivated with an initial OD\textsubscript{600} of 10 in 50 ml of YP medium with 40 g of either glucose or xylose/liter in 125-ml flasks shaken at 100 rpm and were harvested at an OD\textsubscript{600} of 30. For monitoring transcripts in the respiration-deficient mutant, FPL-YSX3P, cells were first grown on YP medium with glucose and harvested at an OD\textsubscript{600} of 1. The harvested cells were transferred into YP medium with xylose, and then RNA was extracted after 24 h of incubation. Residual sugar concentrations were determined by high-performance liquid chromatography (30). The cells were centrifuged at 4°C for 3 min, washed once in sterilized water, frozen in liquid nitrogen, and kept at −80°C for RNA extraction. For RT-PCR, cells were grown overnight in YP medium with either 4% xylose or 4% glucose as the carbon source. A 125-ml flask with 50 ml of culture was inoculated to an initial OD\textsubscript{600} of 0.1 and grown at 200 rpm at 30°C to a final optical density of 1.3 to 2.2. The cells were collected by centrifugation, washed, and frozen in liquid nitrogen. Concentrations of glucose, xylose, xylitol, xylulose, and ethanol were analyzed by high-performance liquid chromatography (Gilsön, Middleton, Wisc.). Cell growth was monitored by OD\textsubscript{600}.

**Genomewide expression analysis.** GeneChip arrays (Affymetrix, Santa Clara, Calif.) were used to monitor mRNA transcripts of putative S. cerevisiae open reading frames. Quantitative RT-PCR was used to measure expression of \textit{P. stipitis} \textit{XYL1}, \textit{XYL2}, and \textit{XYL3} transcripts. Total RNA from yeast cells was isolated as described by Holstege et al. (27). cDNA was synthesized with a T\textsubscript{7}-(dT)\textsubscript{24} primer (GENSET Corp.). Labeling of RNA transcripts, hybridization, and scanning were performed according to the manufacturer’s instructions. The mRNA copy number per cell was calculated using the hybridization signal obtained from the GeneChip software, assuming that there are 15,000 mRNA molecules per yeast cell (30).

RT-PCR. Cells from four independent cultures were used for each condition. mRNA was extracted following the protocol described by Holstege et al. (27). cDNA was constructed using random oligonucleotides and the Reverse Transcription System kit (Promega). RT-PCR analyses of the samples were done with SYBR Green PCR Master Mix (Applied Biosystems) as recommended by the manufacturer, except that 15 pmol of oligonucleotides and a final volume of 25 µl per reaction were used. Genomic DNA of YSX3 was extracted as described by Jin et al. (31) and used for a standard curve. Actin was used to normalize for mRNA concentration. All data points were done in triplicate. A Student \textit{t} test was used to determine if the samples were statistically significant at a 95% confidence level.

**Data analysis and databases.** The DNA-Chip Analyzer (dChip) program (http://www.dchip.org) was used to analyze data from the GeneChip instrument. One culture condition (glucose under high aeration) was repeated to assess reproducibility and to determine the criteria for comparing mRNA levels under different culture conditions. Of 5,944 genes, the mRNA levels of 120 genes (2%) changed more than twofold between replicates. However, the greatest variations were observed for genes expressed at fewer than two copies per cell. To consider only significant transcript changes, we filtered out genes whose mRNA signals changed less than twofold and that were present at fewer than two copies per cell ([mRNA\textsubscript{cop}][mRNA\textsubscript{cop}]/2 and [mRNA\textsubscript{cop}][mRNA\textsubscript{cop}]/2 > 2 copies). By these criteria, if a transcript was present at more than two copies per cell and if its signal changed at least twofold in intensity, we had a 99.7% assurance that the change was significant. To facilitate easier data mining, we constructed a relational database between the GeneChip data and other on-line databases, such as the Saccharomyces genome database (http://genome-www.stanford.edu/Saccharomyces), the Proteome Database (26), and the Comprehensive Yeast Genome Database at the Munich Information Center for Protein Sequences (http://mips.gsf.de/proj/yeast/CYGID/db/index.html). Hierarchical clustering analysis and visualization were performed using the Cluster and Tree-View programs developed by Eisen et al. (12). Expression data are deposited on the Entrez GEO database (http://www.ncbi.nlm.nih.gov/geo). The series ordered group is GSE835. We mapped our expression data to a compiled transcriptional regulatory network using the methods of Herrgard et al. (22, 23).

**Induction of respiration deficiency.** \textit{S. cerevisiae} FPL-YSX3 (10⁷ cells/ml) was treated with 20 µg of ethidium bromide/ml in YPD (30). The flask was wrapped with aluminum foil and incubated at 30°C for 24 h. The cells were cultivated again in YPD with ethidium bromide and then plated in YPD agar. Following isolation, the absence of respiration activity was verified with a Clark-type oxygen electrode, as described by Jin et al. (29).

### RESULTS

Doubling times were ~2.7 and 8h for cells grown on glucose and 4.7 and 16h for cells grown on xylose under aerobic and oxygen-limited conditions, respectively. For all four conditions, residual sugar was present at the time of harvest. No ethanol was detected under the high-aeration conditions, whereas ethanol was detected under oxygen-limited conditions. Although some small amount of ethanol might have been formed under the high-aeration conditions on glucose, the cells were not carbon limited under the low-aeration, high-carbon, and high-cell-density conditions.

**Transcriptional reprogramming with response to carbon source and aeration change.** Oxygen availability did not significantly control gene expression in cells grown on glucose, whereas it greatly affected expression in cells grown on xylose. Of the 5,944 genes detected under the four different culture conditions, only 290 (5%) were differentially expressed under aerobic and oxygen-limited conditions on glucose. In contrast, 509 genes (8.7%) showed differential expression under aerobic and oxygen-limited conditions on xylose (Table 1). The 785 genes whose mRNA levels changed significantly between any two out of the four culture conditions were classified into their functional categories. Genes involved in energy production changed the most (Fig. 1). More than 40% of all genes involved in energy production changed more than twofold in the glucose oxygen-limited (GOL) versus xylose oxygen-limited (XOL) comparison. Genes involved in amino acid metabolism, translation, stress, and defense also changed significantly. Patterns of expression levels identified genes with similar responses over the four different cultivation conditions (6). Although respiration-related genes were coregulated, cluster analysis did not clearly discriminate genes that function together physiologically. Transcript levels for \textit{MTH1}, encoding a repressor of hexose transport genes (47), and \textit{HAP4}, encoding the CCAAT binding protein (3), increased significantly on xylose under aerobic or oxygen-limited conditions. Overexpression of Hap4p causes cells to enter a hyperrespiratory state (37).

**TABLE 1. Numbers of genes showing > 2-fold differences under different conditions**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Glucose</th>
<th>Xylose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic</td>
<td>Oxygen limited</td>
<td>Aerobic</td>
</tr>
<tr>
<td>Glucose aerobic limited</td>
<td>NA</td>
<td>290 (5.0%)</td>
</tr>
<tr>
<td>Xyloose aerobic limited</td>
<td>NA</td>
<td>396 (6.8%)</td>
</tr>
<tr>
<td>Xyloose oxygen limited</td>
<td>NA</td>
<td>509 (8.7%)</td>
</tr>
</tbody>
</table>

mRNA levels of \textit{XYL1} and \textit{XYL2} changed significantly in response to carbon source (Fig. 2). While these two genes were under the control of the same promoter (TDH1), they exhibited different patterns of expression over the four culture conditions. The mRNA level of \textit{XYL3} was much lower than those of \textit{XYL1} and \textit{XYL2}. Transcription of \textit{XYL3} was driven by its native \textit{P. stipitis} promoter, which does not have cis-acting
regulatory sequences native to *S. cerevisiae* and is considerably weaker than the *TDH1* promoter in *S. cerevisiae* (30).

**Engineered *S. cerevisiae* increases transport, TCA, and gluconeogenic transcripts on xylose.** Transcript levels of most genes did not change significantly with respect to carbon sources (Fig. 3), even though glucose was metabolized much faster than xylose. Expression of *HXK1* increased >7-fold when cells were grown on xylose, regardless of aeration conditions. Hexokinase PI (Hxk1p) is induced when cells are grown on nonfermentable carbon sources (21), which suggests that recombinant *S. cerevisiae* recognizes xylose as a nonfermentable carbon source. Clearly, the mRNA levels of genes encoding the tricarboxylic acid (TCA) cycle and respiration pathway enzymes increased during xylose metabolism, and they were induced to a greater extent under oxygen-limited
rather than fully aerobic conditions. These genes were repressed regardless of the aeration conditions during glucose metabolism. The expression of the one exception, CIT2, which codes for citrate synthase, was relatively higher on glucose. Genes for the pentose phosphate pathway were mostly unchanged, except for TKL2, which was induced many-fold on xylose from a low level, and GND2, which was induced on xylose under oxygen-limited conditions. Transcripts for the pentose phosphate pathway were induced 3.4-, 4-, 3.3-, and 7-fold, respectively, when using glucose under oxygen limitation; X/OL, xylose under oxygen limitation.

The expression of the one exception, CIT2, which was induced many-fold on xylose, as well as aerobic conditions. This suggests that NAD + /NADH shuttle systems-particularly mitochondrial internal NADH dehydrogenase (NDH1). Mitochondrial flavoprotein G3-P dehydrogenase (GUT2). DHP, dihydroxyacetone phosphate; G/A, glucose under high aeration: X/A, xylose under high aeration; G/OL, glucose under oxygen limitation; X/OL, xylose under oxygen limitation.

TABLE 2. mRNA levels of hexose transporters under different culture conditions

<table>
<thead>
<tr>
<th>ORF/ Gene</th>
<th>mRNA abundance (no. of copies)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>HXT1</td>
<td>G/A 9.2  G/OL 2.7  X/A 0.1  X/OL 0.1</td>
</tr>
<tr>
<td>HXT2</td>
<td>G/A 2.9  G/OL 0.2  X/A 7.5  X/OL 0.9</td>
</tr>
<tr>
<td>HXT3</td>
<td>G/A 8.2  G/OL 5.5  X/A 0.8  X/OL 1.0</td>
</tr>
<tr>
<td>HXT4</td>
<td>G/A 0.2  G/OL 1.8  X/A 0.1  X/OL 0.7</td>
</tr>
<tr>
<td>HXT5</td>
<td>G/A 0.0  G/OL 0.0  X/A 0.3  X/OL 2.4</td>
</tr>
<tr>
<td>HXT6</td>
<td>G/A 2.2  G/OL 1.8  X/A 31.5  X/OL 50.6</td>
</tr>
<tr>
<td>HXT7</td>
<td>G/A 2.8  G/OL 1.8  X/A 29.1  X/OL 45.0</td>
</tr>
</tbody>
</table>

* ORF, open reading frame.

When we compared the expression analysis data that we obtained in GeneChip studies to those from separate experiments performed with RT-PCR, the two methods led to nearly identical results (Table 3). Where GeneChip studies showed identical results (Table 3). Where GeneChip studies showed consistently higher expression levels for these transporters on GLY than on GLU. In contrast, the expression of the low-affinity transporters was strongly derepressed on xylose, as they are on other nonfermentable carbon sources (34).

Expression of genes for mitochondrial redox shuttle and HAP4. Yeast cells can maintain a neutral redox balance in the cytosol during glucose fermentation by coupling NADH:cytochrome c reductase (complex-I) to the alcohol dehydrogenase (ADH) reaction. This is consistent with previous findings that Hap4p is the main regulator of this complex (15). HAP4 transcript levels were several-fold higher on xylose than on glucose and were highest under oxygen-limited conditions.

When we compared the expression analysis data that we obtained in GeneChip studies to those from separate experiments performed with RT-PCR, the two methods led to nearly identical results (Table 3).
significant differences in transcript levels of cells grown on glucose and on xylose, these same significant differences were detected by RT-PCR. In most instances, however, the magnitudes of the changes appeared to be greater in the RT-PCR experiments. In one case (NDE1), the Affymetrix data did not show a significant difference between the xylose-grown and glucose-grown transcript levels, and the RT-PCR results did show a difference. However, the Affymetrix and RT-PCR changes were each in the same direction (increased expression on xylose), and the significant change indicated by RT-PCR reinforced our overall hypothesis that growth on xylose induces higher levels of respiration-related transcripts.

Redirecting metabolic flux to ethanol by respiration deficiency. Transcript levels clearly indicated that xylose metabolism in recombinant S. cerevisiae was oxidative, because TCA cycle and respiration genes were not repressed by xylose. As a result, metabolic flux at the pyruvate branch point favored respiration over ethanol production. Therefore, we increased the metabolic flux into ethanol production by blocking respiration in S. cerevisiae. Because S. cerevisiae is a petite-positive yeast, a cytoplasmic petite mutant could be isolated by treatment with ethidium bromide. The parental strain (FPL-YSX3) consumed oxygen at a rate of 29.61 ± 1.65 μmol (g of cells·min)−1, while the petite mutant (FPL-YSX3P) did not consume a measurable amount of oxygen (data not shown). We also tested the growth of the petite mutant on glucose and xylose. Interestingly, the YSX3P mutant grew on glucose, but it could not grow on xylose. This result was consistent with previous observations that S. cerevisiae cannot grow anaerobically on xylulose (38) and that xylose-fermenting yeast cannot grow on xylose under anaerobic conditions (49). Transcript analysis of FPL-YSX3P showed that all of the respiration-related genes that were elevated two- to threefold in FPL-YSX3 grown on xylose were down-regulated in the petite mutant. Levels of GND2, HXT5, ADH2, and HXK1 transcripts were higher in FPL-YSX3P than in FPL-YSX3 when both strains were grown on glucose. ADH2 and HXK1 are normally repressed by glucose. When grown on xylose, FPL-YSX3P showed higher levels of HXT5, HXT16, HXT1, and HXK7 than FPL-YSX3. Although the respiration-deficient mutant (FPL-

TABLE 3. Comparison of expression analysis data from Affymetrix and RT-PCR determinations of selected transcripts

<table>
<thead>
<tr>
<th>Gene</th>
<th>Affymetrix</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose †</td>
<td>Xylose †</td>
</tr>
<tr>
<td>ADH5</td>
<td>3.7</td>
<td>1.8</td>
</tr>
<tr>
<td>CDC19</td>
<td>19.2</td>
<td>5.1</td>
</tr>
<tr>
<td>COX5A</td>
<td>8.2</td>
<td>12.6</td>
</tr>
<tr>
<td>FBP2</td>
<td>0.2</td>
<td>1.1</td>
</tr>
<tr>
<td>HAP4</td>
<td>1.7</td>
<td>4.2</td>
</tr>
<tr>
<td>HXK1</td>
<td>1.6</td>
<td>12.5</td>
</tr>
<tr>
<td>HXT1</td>
<td>9.3</td>
<td>0.4</td>
</tr>
<tr>
<td>HXT6</td>
<td>2.4</td>
<td>19.2</td>
</tr>
<tr>
<td>HXT7</td>
<td>2.5</td>
<td>17.3</td>
</tr>
<tr>
<td>MDH1</td>
<td>13.6</td>
<td>28.2</td>
</tr>
<tr>
<td>NDE1</td>
<td>9.9</td>
<td>15.1</td>
</tr>
<tr>
<td>NDH1</td>
<td>2.6</td>
<td>6.2</td>
</tr>
<tr>
<td>PCK1</td>
<td>0.5</td>
<td>1.5</td>
</tr>
<tr>
<td>PGI1</td>
<td>7.7</td>
<td>4.6</td>
</tr>
<tr>
<td>SDH1</td>
<td>6.0</td>
<td>17.4</td>
</tr>
<tr>
<td>TAL1</td>
<td>10.1</td>
<td>10.8</td>
</tr>
<tr>
<td>TKL1</td>
<td>9.8</td>
<td>7.8</td>
</tr>
<tr>
<td>XYL1</td>
<td>ND†</td>
<td>ND†</td>
</tr>
<tr>
<td>XYL2</td>
<td>ND†</td>
<td>ND†</td>
</tr>
<tr>
<td>XYL3</td>
<td>ND†</td>
<td>ND†</td>
</tr>
</tbody>
</table>

† Estimated numbers of transcript copies per cell (see Materials and Methods for calculation).
‡ Difference between glucose- and xylose-grown cells for Affymetrix data with significance determined as explained in Materials and Methods.
§ Estimated number of transcripts per sample normalized to the relative amount of actin (see Materials and Methods for calculation).
¶ Difference between glucose- and xylose-grown cells for RT-PCR data with significance from standard deviation of replicate samples.
¶ ND: not determined; RT-PCR could not distinguish between HXT6 and HXT7 transcripts.
# ND: not determined; Affymetrix GeneChip did not include transcripts for these P. stipitis genes.
YSX3P) could not grow on xylose, it showed improved fermentation capacity relative to its parental strain and produced more ethanol from a mixture of glucose and xylose (Table 4). The maximum ethanol concentration was 1.3-fold greater with FPL-YSX3P than with FPL-YSX3 (Fig. 6). Petite cells that were pregrown on glucose showed specific ethanol production rates on xylose more than three times higher than those of the parental strain (0.043 versus 0.013 g of ethanol g of cells$^{-1}$ h$^{-1}$). The mutant produced more ethanol and accumulated less xylitol from xylose. The maximum ethanol concentrations produced from 40 g of xylose/liter were 5.4 and 10.7 g of ethanol/liter for FPL-YSX3 and FPL-YSX3P, respectively. The ethanol yield increased significantly (from 0.12 to 0.29 g of ethanol/g of xylose), and the xylitol yield decreased slightly (from 0.55 to 0.46 g of xylitol/g of xylose).

Integrating gene expression data into the known regulatory network in yeast. To investigate whether our expression data are compatible with known regulatory effects in yeast and to summarize the data, we superimposed our expression results onto a network of interactive effectors, regulators, and enzymes. We obtained a physical-interaction network with 311 elements from Herrgård et al. (23). This represents a known metabolic network, including small molecule effectors, environmental factors, transcriptional activators, and the genes that they regulate (Fig. 7). We found moderately good correlation between our expression data and the interactions described in the network. Respiration-related transcriptional activators and the genes regulated by these transcriptional factors were expressed more when cells were grown on xylose than when they were grown on glucose. Most transcriptional factors and genes related to amino acid synthesis were expressed more when cells were grown on glucose. In contrast, the GCN4 transcript level was slightly higher on xylose than on glucose. This confirms previous findings that regulation of Gen4p occurs at the level of translation rather than transcription (24).

DISCUSSION

Our experiments were designed to test the effects of two carbon sources and two aeration conditions on the expression profile of engineered S. cerevisiae. To accommodate the physiological changes associated with the different capacities of the cells, other experimental variables were also altered. Cell densities were lower under the high-aeration conditions and higher under the low-aeration conditions to achieve oxygen saturation for full respiration and oxygen limitation for fermentation, respectively. Glucose and xylose concentrations were higher under low aeration to accommodate the higher sugar uptake associated with fermentation (30). While these changes introduced additional variables, the dominant effect was to lower the specific oxygen uptake rate under the low-aeration, high-cell-density conditions. We harvested cells in mid-growth phase under each of the four conditions. Oxygen transfer limits cell growth at very high cell densities. van Hoek et al. showed that with S. cerevisiae the fermentative capacity of the cells correlates strongly with the growth rate (55). One might therefore expect to see lower ethanol production rates because growth rates were lower under the low-aeration, high-cell-density conditions. We observed higher
growth rates under the fully aerobic conditions than under the oxygen-limited conditions, yet we observed ethanol only under oxygen limitation. These results are consistent with higher sugar uptake as a consequence of higher aeration. It is possible that the low cell densities used under high aeration simply did not generate detectable levels of ethanol. While the cell densities differed by 30-fold between the aerobic and oxygen-limited conditions, even at the highest cell density (OD$_{600}$ of 30 ~ 7.5 g [dry weight] of cells/liter) the medium was not limiting growth, so the dominant effect observed was the comparison of fully aerobic growth with oxygen-limited respirofementative growth.

*Saccharomyces cerevisiae* engineered for xylose metabolism clearly did not exhibit a fermentative response to the sugar even under oxygen-limited conditions. In fact, transcript levels for HAP4 and the respiratory proteins that it regulates increased on xylose even as oxygen availability decreased. Many previous studies have examined global transcript levels after environmental and cellular perturbations of *S. cerevisiae*. These include the diauxic shift (9), galactose induction (46), aerobic-anaerobic cultivation (52), mutations in transcriptional apparatus (27), and loss of mitochondrial function (14). Most have used glucose or some carbon sources that are naturally metabolized. Here, we report the expression response of *S. cerevisiae* harboring a complete nonnative xylose metabolic pathway from *P. stipitis*. Even though the capacity for xylose assimilation was functional, the regulatory network for xylose fermentation was not adequate. It is not clear whether this was due to the absence of
specific signal pathways or to other more general regulatory mechanisms. We conclude that when engineering novel metabolic capacity in a heterologous host, it is not sufficient to provide the enzymes for a particular pathway; the rest of the metabolic system must function in a coordinated manner as well.

Two other research groups have recently published papers describing transcriptional profiles in recombinant S. cerevisiae during xylose metabolism. Wahlbom et al. (58) used chemostats to cultivate cells for mRNA measurements. They compared the levels of transcripts of S. cerevisiae TMB 3399 and TMB 3400 when grown on glucose, glucose plus xylose, or (for TMB 3400) xylose alone. Sedlak et al. (48) examined batchwise fermentation of glucose-xylose mixtures by S. cerevisiae 424A(LNH-ST). The present study performed batch fermentations with S. cerevisiae YSX3 using either glucose or xylose alone. Although the other two data sets are not published on line to enable complete comparisons, all three studies found that genes coding for glycolytic enzymes were not significantly affected by the carbon source and that the mRNA levels of XK51, coding for endogenous xylulokinase, were higher in cells grown on xylose alone, whereas they were repressed in the presence of glucose either alone or when present along with glucose. Wahlbom et al. (58) and the present study showed that genes for gluconeogenesis and the glyoxylate pathway (PKC1 and ICL1) are highly expressed in cells grown on xylose. Both groups also reported that transcripts for galactose metabolism were derepressed in the TMB 3400 cells grown on xylose alone. There are also some discrepancies. For instance, in the chemostat studies of Wahlbom et al. (58), the transcript level of HXK1 was higher in cells grown on glucose than in those grown on xylose, whereas in our present studies using batch fermentation, the transcript level of HXK1 was much higher on xylose than on glucose (48). In a glucose-limited chemostat, where the glucose concentration is very low, cells could be in a glucose-derepressed state. Thus, the glucose-limited chemostat culture experiment might not have monitored derepression of HXK1 during the transition of carbon sources from glucose to xylose. In contrast, our cells, which were grown on glucose in batch culture, were under glucose-repressed conditions.

Recently Belinchon and Gancedo showed that xylose could cause moderate carbon catabolite repression in S. cerevisiae TMB3001, a strain that has been engineered for xylose metabolism (2). In that study, growth on 1% xylose induced NAD-dependent glutamate dehydrogenase—24-fold over the level attained with 2% glucose. Activities of fructose-1,6-bisphosphate and isocitrate lyase on 2% xylose were significantly lower than those attained with growth on ethanol but much higher than those seen with growth on glucose. These results were consistent with our findings and those of Wahlbom et al. (58) that growth of engineered S. cerevisiae on xylose induces transcripts for gluconeogenesis.

S. cerevisiae expresses the high-affinity transporters Hxt6p and Hxt7p when growing on glucose or fructose but not on galactose or ethanol, and the low-affinity transporter Hxt1p is induced only at high dilution rates or during the initial phases of batch fermentation on glucose (10). Our studies showed that transcripts of HXT6 and HXT7 were strongly induced on xylose but not on glucose and that HXT1 was induced at a much higher level on glucose than on xylose. Our findings are consistent with a role for xylose as a nonrepressing carbon source that does not trigger induction of low-affinity uptake systems.

It is also possible that cells increased production of respiratory transcripts in response to a low level of sugar uptake. By introducing multiple permease genes, Goffrini et al. enabled Kluyveromyces lactis to grow on galactose and raffinose without respiration (17). Ostergaard et al. were able to increase galactose consumption and respirofermentative activity in S. cerevisiae by altering the regulatory network of the cell (42). If this is the case, overexpression of a xylose transporter might reduce the induction of respiration-related transcripts.

We were able to confirm the Crabtree effect at the level of transcription. This regulatory pattern is characterized by a tight repression of TCA cycle enzymes (encoded by ACO1, IDH2, KGD1, SDH1, and MDH1) and respiratory enzymes (encoded by QCR2 and COX5A) by glucose even under aerobic conditions. We also verified the known regulation of gene expression by oxygen. The mRNA levels of Hap4p, a critical component of the transcriptional activator complex Hap2/3/4, increased threefold under aerobic conditions, even with glucose as a carbon source (Fig. 5). We also discovered unexpected changes in mRNA levels under those conditions. For instance, the mRNA levels of HXK1, FPB1, and PCK1 increased significantly when cells were grown on xylose, regardless of aeration. Expression of these genes is known to increase when cells are grown on nonfermentable carbon sources (Fig. 3). Moreover, the expression of TCA cycle enzymes and respiratory enzymes was not repressed by xylose in the same manner as glucose. Combining these results, we can conclude that recombinant S. cerevisiae does not recognize xylose as a fermentable carbon source. This supports the repression hypothesis—that xylose is poorly metabolized into ethanol because it does not repress respiration in the manner of glucose—rather than the induction hypothesis. In contrast, the induction hypothesis—that xylose does not induce the expression of fermentative enzymes—inot supported, because we observed that mRNA levels of fermentative enzymes (encoded by ADH1 and PDC1) did not change in response to the carbon source. Additional experiments will be necessary to determine whether increased sugar transport can reduce the induction of respiratory transcripts.

Another notable result was that the expression of many oxidoreductases using NADH or NADPH as cofactors increased when cells were grown on xylose. mRNA levels of GDH2, encoding glutamate dehydrogenase, and LYS12, encoding homoisocitrate dehydrogenase, increased significantly when cells were grown on xylose. These enzymes might work to alleviate redox imbalance during xylose metabolism. It might be possible to change the intracellular redox balance by overexpressing GDH2; Nissen et al. (40) showed that the product formation pattern could be changed (glycerol to ethanol) under anaerobic conditions by oxidizing surplus NADH and overexpression of GDH2 in a gdh1 mutant.

Genetic approaches to improving xylose utilization have mostly focused on blocking the oxidative and enhancing the nonoxidative phases of the pentose phosphate pathway (28). This reduces xylitol production by decreasing the supply of NADPH, but it also greatly inhibits xylose assimilation. In the approach used here, we blocked the terminal oxidation of...
NADH by respiration, thereby redirecting reductant into ethanol production. However, the respiration-deficient mutant YSX3P (r°) did not grow on xylose despite being able to ferment the sugar at an elevated rate. This observation is consistent with previous reports. Maleszka and Schneider (38) found that S. cerevisiae required oxygen for growth on D-xylose and that petite mutants of S. cerevisiae did not grow on D-xylose. Likewise, S. cerevisiae metabolically engineered with XYL1, XYL2, and XKS1 did not grow on xylose as a sole carbon source under anaerobic conditions (13). Recent studies have shown that mutants of recombinant S. cerevisiae that show some limited growth on xylose under anaerobic conditions can be obtained through continuous culture (50). While this evolutionary engineering approach is promising, the natures of these mutants are still unknown. Despite several attempts, we have not been able to obtain significant growth of the petite mutants on xylose with minimal medium. It is possible that the complete loss of the mitochondrial genome, as occurs in r° mutants, deletes physiological functions other than respiration that are essential for anaerobic growth on xylose. However, the petite strain could sustain viability on xylose, and the specific xylose uptake rate of the mutant was 50% higher than that of its parent (0.081 versus 0.054 g of xylose g of cells⁻¹ h⁻¹). This is one of the essential characteristics of cells metabolically engineered for industrial fermentations.

ACKNOWLEDGMENTS

This research was supported by USDA/NRICGP grant no. 2001-35504-10695 to T.W.J. We express our profound gratitude to Marcus Herrgård for providing the transcriptional regulatory network database.

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