Respiration-Dependent Utilization of Sugars in Yeasts: a Determinant Role for Sugar Transporters

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Received 9 July 2001/Accepted 16 October 2001

In many yeast species, including *Kluyveromyces lactis*, growth on certain sugars (such as galactose, raffinose, and maltose) occurs only under respiratory conditions. If respiration is blocked by inhibitors, mutation, or anaerobiosis, growth does not take place. This apparent dependence on respiration for the utilization of certain sugars has often been suspected to be associated with the mechanism of the sugar uptake step. We hypothesized that in many yeast species, the permease activities for these sugars are not sufficient to ensure the high substrate flow that is necessary for fermentative growth. By introducing additional sugar permease genes, we have obtained *K. lactis* strains that were capable of growing on galactose and raffinose in the absence of respiration. High dosages of both the permease and maltase genes were indeed necessary for *K. lactis* cells to grow on maltose in the absence of respiration. These results strongly suggest that the sugar uptake step is the major bottleneck in the fermentative assimilation of certain sugars in *K. lactis* and probably in many other yeasts.

*Kluyveromyces lactis* and many other yeast species can grow on galactose and certain oligosaccharides (such as raffinose and maltose) aerobically, but they cannot grow on these sugars anaerobically or in the absence of respiration (15, 18, 19, 35). Assimilation of these carbon sources occurs only under respiring conditions. The phenomenon has been known by the classical name of the Kluyver effect. The kind of sugars involved varies depending on the species and sometimes on the strains within a species. Although the reason for this apparent dependence on respiration for the assimilation of certain sugars is not clear, the phenomenon does appear to be brought about by the interplay of several factors involving lowered rate of transport and metabolism of certain sugars (4). *Saccharomyces cerevisiae* generally does not show this phenomenon (Kluyver effect negative), although *K. lactis* and *S. cerevisiae* seem to use similar pathways to metabolize galactose, raffinose, and maltose.

In the present work we show that, by introducing additional *Saccharomyces* sugar permease genes, *K. lactis* cells can be released from their dependence on respiration for the assimilation of galactose and raffinose. High dosages of both permease and maltase genes were indeed necessary for *K. lactis* cells to grow on maltose in the absence of respiration.

**MATERIALS AND METHODS**

**Strains, media, and growth conditions.** *K. lactis* strains used in this study were PMI-4B (MATa ade1 ade2 ura4), PMI-11B (MATa metA ura4 lys arg rag2), P11-1D (ura4 lys his2-2 lac12-230), P11-1A (lys his2-2 LAC12), 11D304 (MATa his2-2 lac12-230 [32]), JBD100 (MATa trp1 ura3-100 lac4-1), JBD100/M3 (MATa trp1 ura3-100 lac4-1 cyt1 [16]), and J16 (MATa ade1 ade2 trp1 ura3 [9]). The P11-1D lac12 mutant was obtained by sporulation of the P11 diploid constructed by crossing the original mutant 11D304 lac12 (32) with a ura3 LAC12 strain, PMI-11B. As described for the 11D304 lac12 strain (32), the lac12 mutant P11-1D is able to grow on galactose (data not shown).

Genetic procedures for crossing and sporulation have been described previously (18, 40). Rich medium was 1% (wt/vol) Bacto-yeast extract and 2% Bactopeptone (YP). Mineral medium contained 6.7 g of yeast nitrogen base (Difco) per liter without amino acids (YNB) supplemented with the appropriate amino acids and bases. Various carbon sources were added at 2%. Media were solidified with 20 g of Bacto agar per liter. Incubations were done at 30°C. For respiration-dependent growth, the cultures were grown on a reciprocating shaker at 110 rpm.

*The Escherichia coli* strains JM83 [ura Δlac-proAB] rpmL (ΔstrA) Δ800 lacZAM15] was used for plasmid amplification by a standard procedure (33).

**Fermentation test.** As a rapid test to determine the presence or absence of fermentation, fermentation basal medium test (44) was used with minor modifications: production of carbon dioxide leads to a change in color of the indicator dye bromothymol blue (BTB). Precultures, grown for 2 days in liquid minimal medium supplemented with 2% glucose and appropriate auxotrophic requirements, were harvested and diluted at 2 × 10⁶ cells/ml in YP plus galactose (YPGal) liquid medium containing 6 ml of BTB (stock solution: 400 mg of BTB, 1.5 g of NaOH, and H₂O to a final volume of 100 ml, brought to pH 6.5). The cell suspensions were maintained at 28°C for 2 days. The pH indicator dye BTB in the medium turns green to yellow when active fermentation occurs, producing CO₂.

**Plasmids and genomic library.** The gene vectors and plasmids are described in Table 1. All plasmids contained a URA3 marker and a replication origin for *K. lactis*. The *K. lactis*-S. cerevisiae shuttle library of *S. cerevisiae* genomic DNA (30) contained Sac3AI partial digests of *S. cerevisiae* DNA inserted into the yeast shuttle vector pSK1 (11), which can replicate in both yeasts. *K. lactis* transformation was carried out according to Bianchi et al. (5).

**DNA sequencing and sequence analysis.** Sequencing was performed by the dyeodeoxy chain termination method (34) with Sequenase version 2 (USB). Sequence analysis was performed with the BlastP program (2).

**[^14]C]galactose uptake.** The method of sugar uptake was essentially that described by Bisson and Fraenkel (7). Cells grown in minimal medium supplemented with 2% galactose were harvested at the exponential phase of growth (A₆₀₀ 1 to 2), washed twice with ice-cold 0.1 M potassium phosphate buffer (pH 6.5), and resuspended in the same buffer at an A₆₀₀ of 90. A 60-μl aliquot of cell suspension (about 3.5 mg, dry weight) was used for each measurement. Uptake was initiated by addition of 60 μl of radiolabeled 10 mM galactose (186 Bq/μmol). Aliquots of 27 μl were taken at different time intervals, and uptake was terminated by addition of 5 ml of cold water. Cells were collected and washed on a filter paper before radioactivity was measured with a Tricarb liquid scintillation counter (Packard). [U-[^14]C]galactose was obtained from Amersham Pharmacia Biotech. For uptake measurements in the presence of 2.5 μM antimycin A, the cell suspension was preincubated for 5 min with the inhibitor.
Galactose transport is respiration dependent in K. lactis. Galactose utilization in K. lactis, as in S. cerevisiae, is a galactose-inducible activity. Most of the galactose regulon genes are equivalent in the two species (10, 39, 45). Galactose utilization is respiration dependent in K. lactis. In K. lactis, the equivalent galactose permease is the product of LAC12, which is known as the lactose permease gene (32). Some S. cerevisiae strains or variants cannot grow on galactose when respiration is blocked by antimony A or by a mutation causing respiratory deficiency. The reason for this deficiency has been found to be the presence of an inactive gal2 allele (= imp1) in these strains (1, 14). gal2 mutants still grow slowly on galactose, but this growth is completely abolished when antimony A is added or when the strain is transformed into a petite strain. Normal K. lactis strains show a behavior reminiscent of gal2 strains of S. cerevisiae. K. lactis cannot grow on galactose in the presence of antimony A, as if the LAC12 product were deficient. It is, however, not deficient, since K. lactis grows well on galactose when respiration is allowed.

**RESULTS AND DISCUSSION**

**Galactose utilization is respiration dependent in K. lactis.**

Galactose utilization in K. lactis, as in S. cerevisiae, is a galactose-inducible activity. Most of the galactose regulon genes are equivalent in the two species (10, 39, 45). Galactose is first taken up by a permease, which in S. cerevisiae is encoded by GAL2 (29, 36). In K. lactis, the equivalent galactose permease is the product of LAC12, which is known as the lactose permease gene (32). Some S. cerevisiae strains or variants cannot grow on galactose when respiration is blocked by antimony A or by a mutation causing respiratory deficiency. The reason for this deficiency has been found to be the presence of an inactive gal2 allele (= imp1) in these strains (1, 14). gal2 mutants still grow slowly on galactose, but this growth is completely abolished when antimony A is added or when the strain is transformed into a petite strain. Normal K. lactis strains show a behavior reminiscent of gal2 strains of S. cerevisiae. K. lactis cannot grow on galactose in the presence of antimony A, as if the LAC12 product were deficient. It is, however, not deficient, since K. lactis grows well on galactose when respiration is allowed.

**GAL2 gene releases galactose utilization from dependence on respiration in K. lactis.**

We asked whether replacement of LAC12 by GAL2 could render K. lactis cells capable of fermentative growth on galactose. We transformed both LAC12 (PM4-4B) and lac12 (P11-1D) strains of K. lactis with GAL2 (carried either by multicopy or low-copy-number shuttle vectors), and the transformed strains were replica plated onto galactose complete medium (YPGal) containing 5% galactose. We transformed both strains of K. lactis since the gal2 mutant defective in respiration should be incapable of growing on galactose. Indeed, the cyt1 mutant (respirationless due to a mutation of cytochrome c1 [16]) could not grow on galactose (Fig. 1). Again, transformation of this mutant with the GAL2 gene could restore its growth on galactose. Figure 1 presents quantitative aspects of growth on galactose as a function of respiration. The wild type in the presence of antimony A as well as the cyt1 mutant did not grow at all on galactose. The doubling time of the wild-type strain was significantly reduced (3.5 to 3 h) by the introduction of GAL2 plasmids. The wild type with GAL2 plasmids and antimony A as well as the cyt1 mutant with GAL2 plasmids (all respirationless growth) showed similar doubling times of about 10 h. Both the presence of the cyt1 mutation and the addition of antimony A determined a cell mass yield reduced to the same level, as expected for a typical fermentative growth.

**Galactose transport activity is reduced by antimony A and is increased by the addition of the GAL2 gene.**

In K. lactis the utilization of respiratory inhibitors is the only way to analyze the effect of respiratory deficiency on galactose transport. Respiratory mutants are, in fact, unable to grow on galactose and cannot grow even with the addition of low glucose concentrations. Higher concentrations of glucose are not convenient because of the repression of the GAL system. An analysis of the rate of b-[1-14C]galactose transport as a function of respiration was performed in wild-type cells induced by including 2% galactose in the medium and then shifted to buffer containing labeled galactose, in the presence or absence of antimony A (Fig. 2). The drug partially inhibited galactose uptake, leaving about one-third of the activity. In the strain transformed with a low-copy-number GAL2 plasmid, galactose uptake activity was more than doubled, indicating that the GAL2 product was actually contributing to galactose uptake in K. lactis cells. The addition of GAL2 on a high-copy-number plasmid resulted in an increase in galactose uptake activity but at a lower extent than the addition of GAL2 in low copy number. This result suggests that an excess of protein could perturb the equilibrium of the plasma membrane.

The ensemble of results from the galactose experiments

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Structure, properties, and origin (reference)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUK-S11</td>
<td>Multi-copy vector derived from plasmid pKD1</td>
<td>12</td>
</tr>
<tr>
<td>pUK-GAL2</td>
<td>pUK-S11 carrying GAL2 gene (2.6-kb HindIII-EcoRI fragment) (14)</td>
<td>This study</td>
</tr>
<tr>
<td>pUK-MAL2</td>
<td>pUK-S11 carrying K. lactis maltase gene MAL22 (5-kb HindIII-PsiI fragment from pEFHP) (obtained from A. Dominguez, University of Salamanca; unpublished)</td>
<td>This study</td>
</tr>
<tr>
<td>KCp9p1</td>
<td>Centromeric vector (KEp6 + KICEN2), low copy number (&lt;3)</td>
<td>42</td>
</tr>
<tr>
<td>KCpGAL2</td>
<td>KCp9p1 carrying GAL2 gene (3.5-kb HindIII fragment) (14)</td>
<td>This study</td>
</tr>
<tr>
<td>pAF1</td>
<td>pUK-S11 carrying HXT4 gene (3.8-kb XbaI-BglII fragment from clone F)</td>
<td>This study</td>
</tr>
<tr>
<td>pKSI</td>
<td>pUK-S11 carrying HXT1/DDSEI segment (4.0-kb HindIII fragment from clone F)</td>
<td>This study</td>
</tr>
<tr>
<td>pKI</td>
<td>pUK-S11 carrying MAL6T gene (3.7-kb HindIII-BglII fragment) (13)</td>
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</tr>
<tr>
<td>pKL1</td>
<td>KCp9p1 carrying the same MAL6T as above</td>
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</tr>
<tr>
<td>pMM1</td>
<td>pUK-S11 carrying MAL6T-MAL6S segment (7.5-kb HindIII fragment) (13)</td>
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<td>pKM1</td>
<td>KCp9p1 carrying the same MAL6T-MAL6S as above</td>
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supported the idea that in *K. lactis* galactose uptake is the major limiting step for fermentative growth on galactose.

**Additional doses of HXT4 gene release raffinose utilization from dependence on respiration in *K. lactis*.** Raffinose (α-galactosyl-α-glucosyl-β-fructose) is hydrolyzed by the periplasmic invertase to fructose and melibiose (3). The enzyme is encoded in *K. lactis* by *KlINV1*, a homologue of *SUC2* of *S. cerevisiae* (17). Fructose is then transported into the cells by glucose permeases in both species (8, 24, 31, 41). Melibiose is assimilated in *S. cerevisiae* by the product of several *MEL* genes encoding an α-galactosidase (26, 27, 28), whereas this sugar is not assimilated by *K. lactis* (23).

We looked for *S. cerevisiae* genes which might be able to release *K. lactis* from its dependence on respiration for raffinose utilization. We transformed the PM4-4B strain with a *K. lactis-* *S. cerevisiae* shuttle library of *S. cerevisiae* genomic DNA and identified five clones that contained a plasmid with an identical DNA insert of 10 kb. One of them (clone F) was sequenced. The cloned DNA was found to be a segment of chromosome VIII of *S. cerevisiae* (Fig. 3A).

This segment contained the genes *MSRI, HXT4*, and *HXT1* and two unknown open reading frames (ORFs), AHT1 and YHR095W. *HXT4* and *HXT1* are two members of the hexose transporter family. The segment also contained, in the promoter region of the *HXT* genes, DDSE1 and DDSE4 (sequences involved in glucose sensor Snf3p control [37]). Focusing our attention on the *HXT4* and *HXT1* genes and DDSE1 sequence, we subcloned the *HXT4* gene and a DNA fragment including *HXT1* and the DDSE1 region into the *K. lactis* multicopy vector pUK-S11. We named these plasmids pAF1 and pKS1, respectively (Table 1). They were transformed into PM4-4B, and the transformants were tested for growth on raffinose plus antimycin A plates. As reported in Fig. 3A, only the *HXT4* gene was able to support growth in this medium.

The transformation experiment with pAF1 and pKS1 was also performed in the cytochrome c1 mutant JBD100/M3. This respiration-deficient mutant is unable to grow on raffinose. Its growth on raffinose was restored by transformation with the *HXT4*-carrying plasmid (data not shown). This is consistent with the results of the antimycin A experiments. As observed for growth on galactose, the growth curve of the *HXT4*-transformed cyt1 mutant displayed a reduced cell yield on raffinose (optical density at 600 nm [OD 600] of 0.8) with respect to the wild type (OD600 of 4.2), as expected for fermentative growth.

As the *HXT4* gene codes for a hexose transporter, its effect on raffinose utilization was attributed to its supposed permease activity for fructose (a product of raffinose hydrolysis; the other product, melibiose, is not assimilated in this yeast). The increased uptake of fructose might have an additional positive consequence by inducing glycolytic genes like pyruvate decarboxylase (6).

The *HXT4 (=LGT1)* gene of *S. cerevisiae* had been identified as a suppressor of the *rag1* mutation of *K. lactis* (30). The *rag1* mutant, affected in the low-affinity hexose permease gene
and with GAL2 (PM4-4B), transformed or not with GAL2, the mutant is Kluyver effect positive on glucose. HXT4, cannot grow on glucose plus antimycin A (21), that is, GAL2-PM4-4B/KCp-
the mutant gave, however, a reduced cell yield \( \left( A_{600} = 0.7 \right) \), about one-fifth of that of the wild-type strain JBD100 \( \left( A_{600} = 3.6 \right) \).

Altogether, these results suggest that, in K. lactis, the available level of maltose-metabolizing enzymes (permease and maltase) is too low to sustain fermentative growth, which demands a high flow of substrate, while this level is sufficient for respiratory growth.

To conclude, we here demonstrated that additional doses of S. cerevisiae sugar transporter genes can release K. lactis cells from their dependence on respiration for the utilization of galactose, maltose, and raffinose. For galactose and raffinose utilization, the limiting step, in the absence of respiration, is clearly the uptake of the substrate. In the case of maltose, uptake of the substrate is not the only step that limits fermentative growth. In addition to an insufficient permease level, the maltase activity is also too low to ensure fermentative growth.

The K. lactis effect is a widely observed phenomenon in the utilization of various non-glucose sugars by many yeast species. The genetic basis of the phenomenon was examined here on the model of K. lactis with several sugars. The question is of practical importance, since glucose is generally not the main sugar substrate in industrial processes.

One may ask whether lactose utilization by K. lactis is respiration dependent (this yeast and Kluyveromyces marxianus are used for industrial hydrolysis of lactose). A survey on laboratory strains of K. lactis shows that the ability to grow on

**FIG. 2.** Effect of antimycin A on galactose transport. K. lactis cells (PM4-4B), transformed or not with GAL2-carrying monocopy plasmid KCP-GAL2 and with GAL2-carrying multicopy plasmid pUK-GAL2, were grown on galactose, washed, and suspended in phosphate buffer. Uptake radiolabeled galactose was determined in the presence and absence of antimycin A as detailed in Materials and Methods. Time zero values were subtracted from all measurements. Symbols: wild-type PM4-4B in the absence (●) and presence (○) of antimycin A; PM4-4B/KCP-GAL2 in the absence (■) and presence (□) of antimycin A; PM4-4B/pUK-GAL2 in the absence (▲) and presence (●) of antimycin A. The values are means of three independent experiments. In no case was the variation higher than 15%.
lactose plus antimycin A is a strain-dependent property (P. Goffrini, unpublished data), suggesting that the lactose permease activity varies in different strains. The present study suggests that the fermentation capacity on lactose may be increased by specifically amplifying the LAC12 gene. The well-known Kluyver effect for maltose utilization in Candida utilis (22, 43) might also involve a limited maltose transport activity of this yeast.

In the genome of S. cerevisiae, a species which has a preference for fermentative life, the sugar transporter genes are amplified to the extreme, and this yeast is considered Kluyver effect negative, but this is not true for all sugars. For example,

**FIG. 3.** (A) Physical map of the HXT4 gene region of S. cerevisiae chromosome VIII. The region cloned into the K. lactis transformant clone F (see text) is shown. The flanking sequences used as primers for sequencing are indicated with arrows and broken lines. pAF1 and pKS1 are subclones. (B and C) Physical map and subcloning of the MAL regions of S. cerevisiae and K. lactis, respectively. Plasmid pCV-H2 contains S. cerevisiae MalT (maltose permease) and MAL6S (maltase), and plasmid pEFPH contains the K. lactis MAL22 gene (maltase) (A. Dominguez, unpublished data). pKK1, pKL1, pMM1, pKM1, and pUK-MAL22 are subclones. Open reading frames and DDSE sequences are shown by boxes. DNA inserts are represented by thin lines. Restriction sites: B, BglII; H, HindIII; P, PstI; X, XbaI. The + and – signs stand for the presence and absence, respectively, of transformed clones on rafinose plus antimycin A medium (A) and on maltose plus antimycin A medium (B and C).
it is Klyover effect positive for trehalose, probably because of an insufficient level of the trehalase transporter (AGT1 product), as suggested by Malluta et al. (25).

ACKNOWLEDGMENTS

We thank H. Fukuhara for helpful suggestions and for critical comments on the manuscript. We also thank A. Dominguez (Department of Microbiology and Genetics, University of Salamanca, Spain) for the pEFHP plasmid; R. Dickson (Department of Biochemistry, University of Kentucky College of Medicine, Lexington, Ky.) for the Klac-12 mutant 11D304; J. Subik (Department of Microbiology and Virology, Faculty of Natural Sciences, Comenius University, Bratislava, Slovak Republic) for the cytochrome c_{1} mutant JBD 100/MS3; M. Vanoni (Dipartimento di Biotecnologie e Bioscienze, Universita degli Studi di Milano-Bicocca, Milan, Italy) for the pCV-H2 plasmid; and M. Wesołowski-Louvel (Centre de Genétique Moléculaire et Cellulaire, CNRS UMR 5534, Université Claude Bernard, Villeurbanne, France) for the S. cerevisiae genomic library.

This work was supported by grants from the Ministero Universita`e-
repression of the Kluyveromyces lactis invertase gene KlINV1 does not

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