RAG4 Gene Encodes a Glucose Sensor in *Kluyveromyces lactis*

Svätopluk Betina,*[,1] Paola Goffrini,[†] Iliana Ferrero[†] and Micheline Wésolowski-Louvel*

**Unité Microbiologie et Génétique, Université Claude Bernard, 69622 Villeurbanne, France and [†]Istituto di Genetica, Università degli Studi di Parma, 43100 Parma, Italy*

Manuscript received December 11, 2000
Accepted for publication February 19, 2001

ABSTRACT

The *rag4* mutant of *Kluyveromyces lactis* was previously isolated as a fermentation-deficient mutant, in which transcription of the major glucose transporter gene *RAG1* was affected. The wild-type *RAG4* was cloned by complementation of the *rag4* mutation and found to encode a protein homologous to Snf3 and Rgt2 of *Saccharomyces cerevisiae*. These two proteins are thought to be sensors of low and high concentrations of glucose, respectively. Rag4, like Snf3 and Rgt2, is predicted to have the transmembrane structure of sugar transporter family proteins as well as a long C-terminal cytoplasmic tail possessing a characteristic 25-amino-acid sequence. Rag4 may therefore be expected to have a glucose-sensing function. However, the *rag4* mutation was fully complemented by one copy of either *SNF3* or *RGT2*. Since *K. lactis* appears to have no other genes of the *SNF3/RGT2* type, we suggest that Rag4 of *K. lactis* may have a dual function of signaling high and low concentrations of glucose. In *rag4* mutants, glucose repression of several inducible enzymes is abolished.

FERMENTATION is the main mode of energy acquisition in *Saccharomyces cerevisiae*, and the redundancy of many glycolytic genes in this yeast may reflect its importance. By contrast, in *Kluyveromyces lactis* fermentation is dispensable, and glycolytic genes are not redundant. This situation allowed us to isolate many mutants defective in key genes of the fermentation pathway. The central role of glucose transporters in the regulation of fermentation has thus been demonstrated (Chen *et al.* 1992; Wésolowski-Louvel *et al.* 1992a). A number of *trans*-acting elements involved in this regulation have also been identified.

The glucose uptake system in *K. lactis* relies on two, nonredundant, glucose transporters with a few exceptions found in a variant set of strains: a low-affinity carrier encoded by *RAG1* (Wésolowski-Louvel *et al.* 1992a) and a high-affinity carrier encoded by *HGT1* (Billard *et al.* 1996). The expression of *RAG1* is glucose inducible (Chen *et al.* 1992; Wésolowski-Louvel *et al.* 1992a), whereas *HGT1* is constitutively expressed (Billard *et al.* 1996). The expression of *RAG1* is necessary for fermentative growth on high concentrations of glucose, because *rag1* mutants are unable to grow on 5% glucose when respiration is blocked by antimycin A [this is called Rag$^-$ phenotype (resistance to antimycin A on glucose); Goffrini *et al.* 1989]. A series of mutants displaying the Rag$^-$ phenotype have been isolated (Wésolowski-Louvel *et al.* 1992b) and at least three of them carry a mutation in one of the three genes that positively regulate the transcription of the *RAG1* gene (Chen *et al.* 1992): *RAG5*, which encodes the single hexokinase of *K. lactis* (Prior *et al.* 1993); *RAG8*, encoding a caseine kinase I (Blaisonneau *et al.* 1997); and *RAG4*, the subject of this study. *RAG4* belongs to the *SNF3/RGT2* family of genes that, in *S. cerevisiae*, are thought to form part of the glucose-sensing apparatus (Özcan *et al.* 1996, 1998).

MATERIALS AND METHODS

Yeast strains and growth conditions: The strains used in this study are as follows: MW270-7B (*MAT***a** *uraA1-1 leu2 metA1-1* Rag$^+$; Billard *et al.* 1996); PM6-7A/VV32 (*MAT***a** *uraA1-1 adeT-600 rag4-1* Rag$^-$; Wésolowski-Louvel *et al.* 1992b); JA6 (*MAT***a** *ura3 trp1 ade1-600 adeT-600* Rag$^+$; Breunig 1989); MW109-8C/FA49 (*MAT*α *lysA1-1 rag4-5* Rag$^-$; Wésolowski-Louvel *et al.* 1992b); JA6/29-4 [*MAT***a** *ura3 trp1 ade1-600 adeT-600 kht1*(=*rag1*) *kht2* Rag$^-$]; JA6/151 (*MAT***a** *ura3 trp1 ade1-600 adeT-600 rag5* Rag$^-$; Goffrini *et al.* 1995).

Yeast cells were grown at 28° either in a complete medium containing 1% Bacto yeast extract, 1% Bacto-peptone (Difco, Detroit), supplemented with glucose (as indicated) or in a minimal medium containing 0.7% yeast nitrogen base without amino acids (Difco) but with auxotrophic supplements as required and a specified carbon source. The Rag phenotype was tested on GAA medium (5% complete glucose medium supplemented with 5 μM antimycin A). The 2-deoxy-D-glucose-resistant phenotype Dgr$^+$ was tested in a minimal medium containing 2-deoxy-D-glucose (Sigma, St. Louis) at the concentration of 5 g liter^{-1}. The different carbon sources utilized were added at 2% (w/v).

Genetics methods have been described previously (Wésolowski *et al.* 1982; Goffrini *et al.* 1989).

Corresponding author: Micheline Wésolowski-Louvel, Unité Microbiologie et Génétique, ERS 2009 CNRS/UCBL/INSA, Université Claude Bernard, Bâtiment 405, 43, Boulevard du 11 Novembre 1918, 69622 Villeurbanne Cédex, France. E-mail: louvel@univ-lyon1.fr

[1]*Present address:* Cancer Research Institute, Slovak Academy of Sciences, Department of Molecular Biology and Biochemistry, Bratislava 842 15, Slovakia.

Yeast transformation: Replicative transformation of *K. lactis* was performed by electroporation. For integrative transformation of *K. lactis*, the procedure described by Dohmen *et al.* (1991) was followed.

Cloning and sequencing of *RAG4* gene: The *RAG4* gene was cloned by *in vivo* complementation of *rag4-1* mutation (strain PM6-7A/VV32) with a *K. lactis* genomic library made in the KCp491 vector. Out of 13,000 Ura^+ transformants, two were found to be Rag^+. The complementing plasmids extracted from these two transformants were then amplified in *Escherichia coli*. In each case, amplified DNA showed an expected pattern (vector + insert) for only one preparation out of three. In the other cases, the restriction profiles of DNA suggested loss of the insert together with reorganization of the vector. Further large-scale preparation of the complementing DNA turned out to be impossible, whatever the *E. coli* host (MC1066, XL1-Blue, and Sure). The complementing DNA that we had obtained in small quantities allowed us to establish that the two Rag^+ transformants carried the same plasmid pSB1 containing a 6.0-kb insert (Figure 1). To save this DNA preparation, part of the restriction map of pSB1 was obtained from PCR-amplified DNA. The oligonucleotides used were as follows: TetBup, TCCTGCTCGCTTCGCTACTTGG (upstream of the *Bam*HI site of tetracycline marker in which was cloned the genomic DNA of *K. lactis*) and TetBlw, CCATACC CACGCCGAAACAAGC (downstream of the *Bam*HI site). Then, subfragments of the amplified insert were cloned into pCXJ18 vector and tested for their stability in *E. coli* as well as for their ability to complement the *rag4-1* mutation. The results (Figure 1A) indicated that the largest subfragment (4.4-kb *Pvu*II-*Bam*HI) stable in *E. coli* did not complement the *rag4* mutation. Therefore, the 2.7-kb *Pvu*II-*Eco*RI fragment of the pSB1 plasmid (original DNA) cloned into *Sma*I and *Eco*RI sites of pCXJ18 plasmid was entirely sequenced on both strands. The 5′ end of the *RAG4* gene (upstream of the *Pvu*II site) was sequenced after PCR amplification, using the Expand Long Template PCR system (Boehringer, Mannheim, Germany) with pSB1 original DNA as template. The oligonucleotides used were as follows: TetBup (see above) and RG-41, TGACGTGAACGATGTTCA (starting 100 nucleotides downstream of the *Pvu*II site of pSB1 DNA). The resulting 1.8-kb PCR product was sequenced on both strands.

The *RAG4* nucleotide sequence has been assigned the EMBL accession no. Y14849.

Construction of Δ*rag4* deletion strains: The 2.7-kb *Pvu*II-*Eco*RI fragment containing most of the 3′ region of the *RAG4* gene and cloned into the pCXJ18 plasmid was recloned into the pBluescript KS phagemid (Stratagene, La Jolla, CA), using the *Bam*HI and the *Eco*RI sites (Figure 1B). The resulting plasmid was digested with *Sty*I, blunt ended with Klenow enzyme prior to digestion with *Bgl*II. Then, the internal 1.5-kb *Sty*I-*Bgl*II fragment was replaced by a 1.0-kb *Sph*I-*Eco*RI fragment that contained the *URA3* marker from pAF101 vector (provided by B. Dujon, Institut Pasteur, Paris; Thierry *et al.* 1990). A 2.2-kb *Bam*HI-*Eco*RI fragment that contained the disrupted *RAG4* cassette was used to transform two Rag^+ hosts MW270-7B and JA6 to uracil prototrophy. Correct integration of the disrupted gene was verified by Southern hybridization (data not shown).

Complementation of the *rag4* mutation by *SNF3* and *RGT2*: The two genes were cloned in low-copy-number *URA3*-marked vectors of *K. lactis*. The 4.1-kb *Hin*dIII-*Nru*I fragment of the *SNF3*-containing plasmid pBL8 (provided by M. Carlson, Columbia University, New York; Marshall-Carlson *et al.* 1990) was cloned into the *Hin*dIII-*Nru*I site of KCp491 (Prior *et al.* 1993). In the case of *RGT2*, a 3.3-kb *Eco*RI-*Bam*HI fragment of plasmid pBM3272, provided by S. Özcan (Washington University, St. Louis), containing the entire *RGT2* gene was cloned into the *Eco*RI-*Bam*HI site of pCXJ18 (Chen 1996). The constructs were individually transformed into the original *rag4-1* strain (PM6-7A/VV32). The Ura^+ transformants thus obtained were then tested for their Rag phenotype by replica-plating on 5% glucose + antimycin A (GAA) plates.

Preparation of yeast RNA and probes: Total RNA was extracted from cells grown to an OD_{600} of 2 to 3. $Poly(A)^+$ enriched mRNA were obtained using an mRNA separator (CLONTECH, Palo Alto, CA). The *RAG4* probe used was the 0.98-kb *Pvu*II-*Kpn*I fragment (Figure 1) of pSB1 DNA. The *RAG1* probe used was a specific 0.9-kb *Sal*I-*Pst*I internal fragment of the gene (Wésolowski-Louvel *et al.* 1992a). This probe that was also used to analyze *KHT1* (identical to *RAG1*) and *KHT2* (a variant of *RAG1*) transcription in JA6 strain does not discriminate between the two transcripts. The *HGT1* probe was a 1.75-kb *Eco*RI-*Hin*dIII fragment containing the *HGT1* gene (Billard *et al.* 1996). *KlGAL80* and *LAC9* probes were obtained by PCR amplification using *K. lactis* genomic DNA as template. The oligonucleotides used were as follows: LAC9a, ATGGGTAGTAGGGCCTCCAATTCG; LAC9b, CACTGTTC GTACCAGTGGTCTC; GAL80a, CGGCAGGACGGCATCAT CATGAAC; and GAL80b, GAGGACATGGCAACATTAAG. The *LAC4* probe used was the plasmid pLX8Δ (provided by K. D. Breunig, Martin-Luther-University, Halle-Wittenberg, Germany) containing the whole *LAC4* gene. *KlCYB2* probe was a 1.9-kb *Sal*I-*Eco*RI fragment from p30 plasmid (Alberti *et al.* 2000). In all cases a specific probe of *K. lactis* actin gene was used in parallel as a quantitative reference.

Measurement of glucose uptake: Glucose uptake was measured as previously described (Billard *et al.* 1996). Uptake activity was determined by a 10-sec incubation with a ^{14}C-labeled glucose at each glucose concentration.

Preparation of cell-free extract and enzyme assays: Cells, harvested at a density of $\sim 5 \times 10^7$ cells ml^{-1}, were resuspended in extraction buffer (0.1 M Tris-HCl, pH 8.5, 1 mM phenylmethylsulfonyl fluoride) and disrupted by vortexing at 4° in the presence of glass beads. The supernatant was utilized for measurement of the enzyme activity.

β-Galactosidase (EC 3.2.1.23) activity was assayed as described by Cohn and Monod (1951), and L-lactate ferricytochrome c-oxidoreductase (L-LCR; EC 1.1.2.3.) according to Lodi *et al.* (1994). Total α-glucosidase (maltase, EC 3.2.1.20 and isomaltase, EC 3.2.1.10) activity was tested with *p*-nitrophenyl-*O*-D-glucopyranoside as a substrate as described by Zimmermann *et al.* (1977). The Lowry method was used for protein quantification with bovine serum albumin as standard.

RESULTS

Isolation of the *RAG4* gene and deduced amino acid sequence of its product: We isolated a plasmid (pSB1) from a CEN-based *K. lactis* genomic library by its ability to complement the *rag4-1* mutation and confer the Rag^+ phenotype (see MATERIALS AND METHODS). The nucleotide sequence of the left end of the 4.4-kb *Pvu*II-*Bam*HI fragment revealed the presence of an open reading frame (ORF) whose putative product showed a high homology with *SNF3* and *RGT2* gene products of *S. cerevisiae*. This ORF should correspond to the *RAG4* gene since the right half of the insert was not complementing. The complete nucleotide sequence of the *RAG4* gene was established as reported in MATERIALS AND METHODS. The deduced Rag4 protein was found to be 716 amino acids long, of which the first 113 were

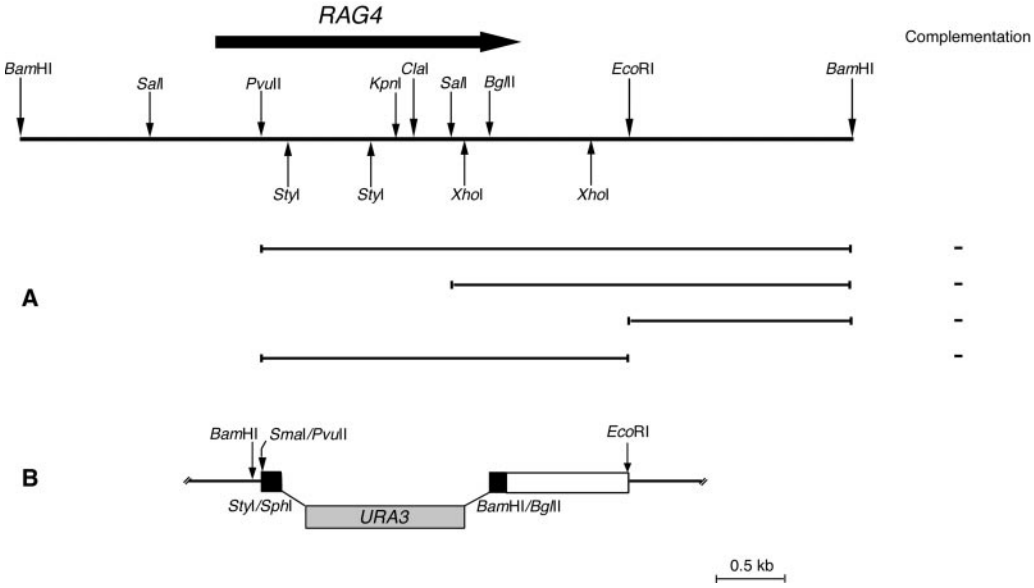

FIGURE 1.—(A) Restriction map of recombinant plasmid pSB1 carrying the *RAG4* gene and other subclones. →, open reading frame; + and −, presence or absence of complementation of *rag4* mutation. (B) Internal fragment disruption of the *RAG4* gene with *URA3* marker (shaded box). The open and solid boxes indicate the genomic fragment. Solid boxes represent the *RAG4* sequence (see MATERIALS AND METHODS).

localized to the left of the *Pvu*II site of pSB1 DNA (Figure 1). Probably it is this or flanking region of the *RAG4* gene that, for unknown reasons, contained the element(s) of instability (see MATERIALS AND METHODS). The predicted Rag4 protein presents 12 potential transmembrane domains, typical of sugar permeases, as well as a 251-residue C-terminal tail. This C-terminal extension is characteristic of the glucose sensor proteins of *S. cerevisiae* Snf3 and Rgt2, which have been shown to control the transcription of several glucose permease genes (LIANG and GABER 1996; ÖZCAN *et al.* 1996). Rag4 has the same level of similarity (72–74%) and identity (52–53%) with both proteins. The highest level of identity between these three proteins is localized within the transmembrane regions (63–66%). In the three proteins, the carboxy-terminal extensions are rather dissimilar, except for a sequence motif of 25 amino acids that occurs twice in Snf3 and only once in Rgt2 and Rag4. This sequence has been shown to be essential for the signaling function in *S. cerevisiae* (ÖZCAN *et al.* 1998; VAGNOLI *et al.* 1998).

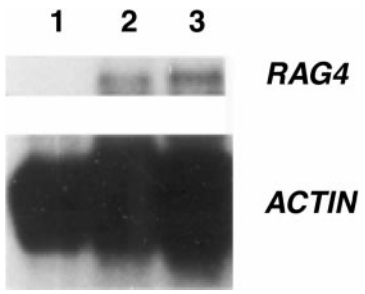

FIGURE 2.—Northern blot analysis of *RAG4* mRNA. Each slot was loaded with ~5 μg of poly(A)$^+$ RNA and electrophoresed on a 1.2% agarose-formaldehyde gel. The probes used are described in MATERIALS AND METHODS. Lane 1, MW270-7B/Δ*rag4* null mutant strain grown on 2% glucose; lane 2, MW270-7B (*RAG4*) grown on 2% glycerol; lane 3, MW270-7B (*RAG4*) grown on 2% glucose.

Disruption and expression of *RAG4* gene: A *rag4* null mutant is Rag$^-$. Moreover, the allelism of the disrupted gene with *rag4* was confirmed by the absence of complementation in a cross between the disruptant and the *rag4-5* strain (MW109-8C/FA49). Thus, the cloned gene indeed corresponds to the *RAG4* locus, and the *rag4* null mutation is not lethal.

Transcript level of *RAG4* was found to be very low, and the mRNA could be detected only when the poly (A)$^+$ fraction of the total RNA was used (Figure 2). *SNF3* and *RGT2* genes of *S. cerevisiae* are also known to be transcribed at low level (NEIGEBORN *et al.* 1986; ÖZCAN *et al.* 1996). The *RAG4* transcript is 2.8–2.9 kb long, consistent with the size of the ORF. The level of *RAG4* mRNA did not change significantly with the carbon sources of the growth media (2% glycerol or 2% glucose; Figure 2).

Complementation of the *rag4* mutation by *RGT2* and *SNF3* genes of *S. cerevisiae*: The functional similarity of Rag4 and Snf3/Rgt2 was suggested by the impairment of transcription of glucose permease genes in the corresponding mutants: *RAG1* gene transcription is affected in *rag4* mutant in *K. lactis* (CHEN *et al.* 1992), as is transcription of several *HXT* genes in *snf3* and *rgt2* mutants of *S. cerevisiae* (ÖZCAN *et al.* 1996). The similarity of the Rag4 protein to Snf3 and Rgt2, as well as the similar effect of *rag4* and *snf3* and *rgt2* on expression of glucose transporter genes prompted us to try to complement the *rag4* mutation with the *SNF3* and *RGT2* genes. Each of the two genes was able to restore growth of the *rag4* strain on glucose + antimycin A (Figure 3). This was unexpected since *SNF3* and *RGT2* have distinct sensor functions for glucose.

Growth phenotype and glucose uptake in the *rag4*

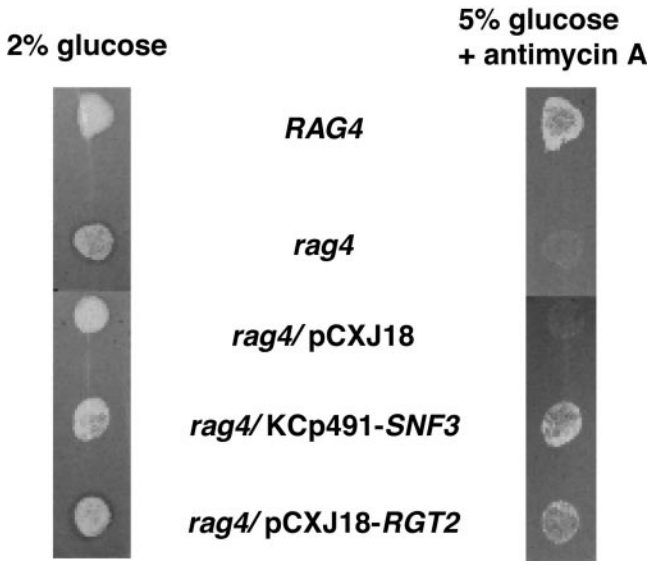

FIGURE 3.—Complementation of the *rag4* mutation by *RGT2* and *SNF3* genes of *S. cerevisiae*. *rag4* cells (strain PM6-7A/VV32) transformed with the centromeric vector pCXJ18 (control) or with the *SNF3* and *RGT2* genes carried by KCp491 and pCXJ18, respectively. The transformants were grown on 2% glucose uracil-less medium prior to being replica plated onto GAA medium. The growth on GAA plates (Rag^+/Rag^- phenotype) was checked after 24 hr of incubation at 28°. *RAG4* strain (MW270-7B) was used as a Rag^+ control.

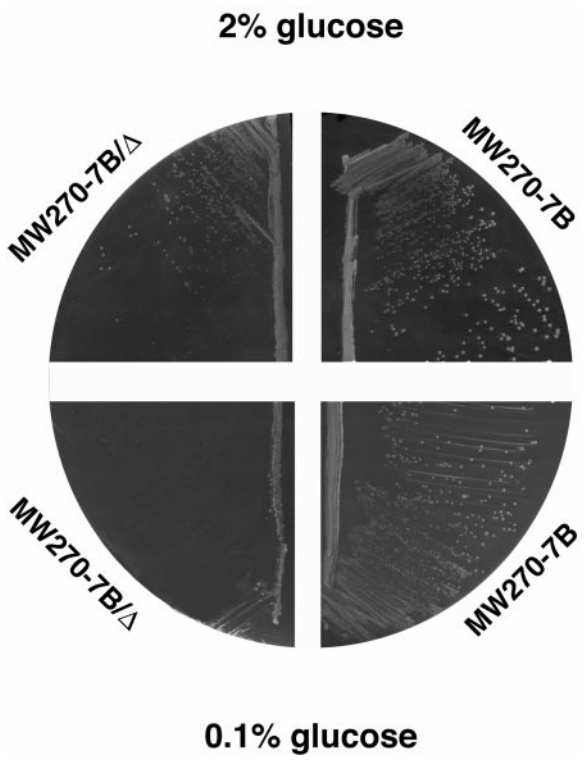

FIGURE 4.—Growth phenotype of the *rag4* null mutant strain. The two strains MW270-7B (*RAG4*) and MW270-7B/Δ*rag4* (*rag4::URA3*) were streaked to single colonies on 0.1 and 2% glucose minimal plates and incubated for 3 days at 28° before the photographs were taken.

null mutant: The *S. cerevisiae snf3* mutant but not *rgt2* is defective in growth on raffinose (low glucose) plus antimycin (NEIGEBORN *et al.* 1986; SCHMIDT *et al.* 1999). The *snf3 rgt2* double mutant does not grow on raffinose or glucose (0.2 and 2%) plates containing antimycin A and grows poorly on 2% glucose even in the absence of antimycin A (ÖZCAN *et al.* 1998; SCHMIDT *et al.* 1999). We examined in more detail the growth of the *rag4* null mutant on low glucose (0.1%) and high (2%) glucose, as compared to its isogenic wild-type strain. Figure 4 shows that the growth of the *rag4* null mutant was reduced at both concentrations of glucose. The phenotype of the *rag4* mutant on glucose thus appears to be similar to that of the *snf3 rgt2* double mutant of *S. cerevisiae*.

In the *rag4* mutant, the glucose-induced expression of the low-affinity glucose permease gene *RAG1* is severely reduced (CHEN *et al.* 1992; this study). We therefore measured the actual glucose uptake activities in the *rag4* null mutant. As shown in Figure 5, both low- and high-affinity glucose transport activity is reduced in the mutant, although not completely absent. Therefore, the reduced growth rate on glucose of the *rag4* mutant may be explained by its low glucose uptake activity.

The expression of glucose-permease genes is controlled by Rag4: As in the original *rag4* strain, the transcription of *RAG1* could not be detected in the null mutant (Δ*rag4*), while that of *HGT1* was slightly increased in the mutant (Figure 6, A and B). Most *K. lactis* strains harbor the two single genes *RAG1* and *HGT1* coding for low- and high-affinity glucose permeases, respectively. However, some natural isolates do not contain the *HGT1* gene (our unpublished data), and their *RAG1* locus is replaced by two tandemly arranged glucose transporter genes, *KHT1* (identical to *RAG1*), inducible by high levels of glucose, and *KHT2* (a variant of *RAG1*), weakly induced by low glucose and repressed by high glucose (WEIRICH *et al.* 1997; BREUNIG *et al.* 2000). In this type of strain, the transcripts of both genes are practically absent in Δ*rag4* mutant cells grown either at low or high glucose concentration (Figure 6C). Therefore, Rag4 appears to be required for the specific glucose-induced expression of both of these genes. It is noteworthy that, in this case, induction by both low and high concentrations of glucose is dependent on Rag4.

Role of *RAG4* gene in glucose repression: The *K. lactis* strains most sensitive to glucose repression are those mentioned above that contain the two glucose transporter genes *KHT1* and *KHT2* (GOFFRINI *et al.* 1995; BREUNIG *et al.* 2000). Therefore, we used the JA6 wild-type strain and its isogenic derivatives to investigate the possible role of Rag4 in glucose sensing and glucose repression. To measure glucose repression, we tested the ability of 2-deoxy-D-glucose (2-DOG), a nonmetabo-

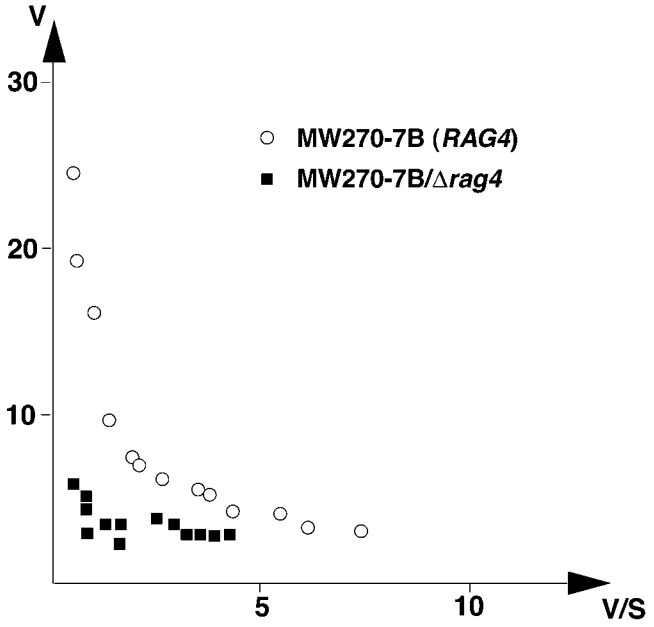

FIGURE 5.—Glucose uptake in *rag4* null mutant. At various concentrations of glucose (0.5–80 mM), the rate of D-[^{14}C]glucose uptake was determined by a 10-sec incubation. Uptake velocity, *V*, expressed as nanomoles of glucose per milligram (dry cell mass) per minute, was plotted against *V*/*S* (*S* is the millimolar glucose concentration).

lizable glucose analogue known to cause glucose repression, to inhibit growth on galactose, maltose (α-glucosyl-α-glucose), raffinose (α-galactosyl-α-glucosyl-β-fructose), ethanol, and lactate (ZIMMERMANN and SCHEEL 1977; GOFFRINI *et al.* 1995). As shown in Figure 7, the *rag4* mutant, but not wild type, was able to grow in the presence of the 2-DOG on lactose, galactose, lactate, and ethanol. Figure 7 also shows that both a *kht1 kht2* mutant and a *rag5* (hexokinase) mutant grew on all these carbon sources in the presence of 2-DOG. These results suggest that *rag4* mutation causes a release from glucose repression of the enzymes involved in the utilization of lactose, galactose, lactate, and ethanol, but not the enzymes of maltose or raffinose metabolism.

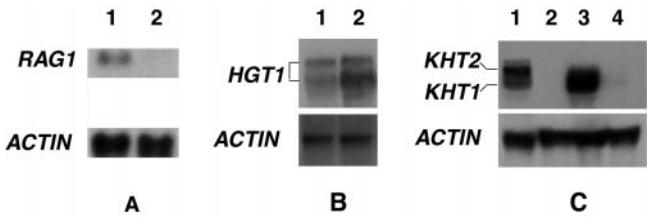

FIGURE 6.—Effect of the disruption of *RAG4* on the transcription of glucose-permease genes. Approximately 15 or 25 μg of total RNA extracted from cells grown on glucose complete medium was loaded in each slot. Electrophoresis conditions were as in Figure 2. The probes used are described in MATERIALS AND METHODS. (A) *RAG1* transcription. The cells were grown on 2% glucose medium. MW270-7B (*RAG4*) strain (lane 1); isogenic MW270-7B/Δ*rag4* strain (lane 2). (B) *HGT1* transcription. The cells were grown on 2% glucose medium. MW270-7B (*RAG4*) strain (lane 1); isogenic MW270-7B/Δ*rag4* strain (lane 2). (C) *KHT1* and *KHT2* transcription. Total cellular mRNA was prepared from cells grown on 0.2% (lanes 1 and 2) and 2% glucose (lanes 3 and 4). JA6 (*RAG4*) strain (lanes 1 and 3); isogenic JA6/Δ*rag4* strain (lanes 2 and 4).

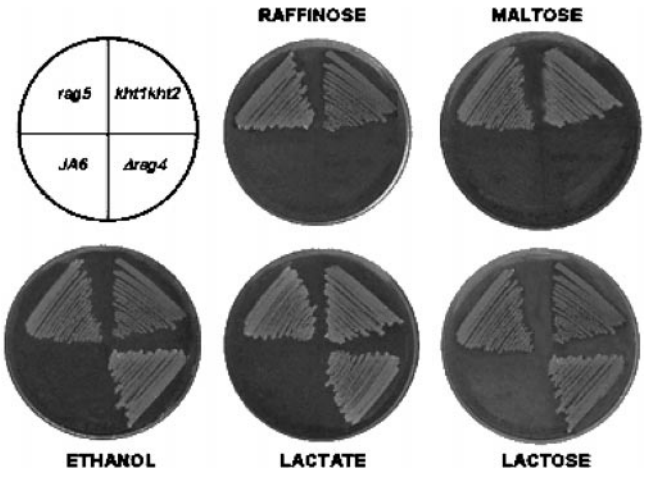

FIGURE 7.—Effect of different *rag* mutations on deoxyglucose resistance phenotype. The strains were streaked on minimal media supplemented with 2% of the different carbon sources and 0.5% 2-deoxy-D-glucose. The plates were incubated for 3 days at 28° before the photographs were taken.

The levels of the enzymes necessary for the utilization of lactose/galactose and L-lactate [β-galactosidase and L-lactate ferricytochrome c-oxidoreductase (L-LCR), respectively] were completely derepressed in cells grown on glucose (Figure 8). Maltase activity was derepressed in the *kht1 kht2* mutant, but not in the *rag4* mutant. These results are fully consistent with the growth phenotypes observed in the presence of 2-DOG.

The derepression phenotypes observed in *rag4* are mediated by changes in the transcription of the corresponding genes, since the levels of *LAC4* and *KlCYB2* mRNA are strongly reduced by glucose in wild type, but not in the *rag4* mutant (Figure 9A).

The effect of Rag4 on glucose repression of *LAC4* is probably mediated through one of the two regulatory genes of the lactose-galactose regulon: *LAC9* (*KlGAL4*), the transcriptional activator (SALMERON and JOHNSTON 1986), and *LAC10* (*KlGAL80*), the inhibitor of *KlGAL4* (DICKSON *et al.* 1981; ZENKE *et al.* 1993). Rag4-dependent repression of the lactose-galactose regulon could result either from a positive effect on the expression of *LAC10* or from a negative effect on the activator gene *LAC9*. The expression of *KlGAL80* was not affected by *rag4* mutation both in repressing (glucose + galactose) and nonrepressing (galactose) conditions (Figure 9B). In galactose medium, *LAC9* gene was transcribed to a similar level in the wild-type and *rag4* strains. When glucose was present in the growth medium, *LAC9* transcription was reduced in the parental strain (*RAG4*) but not in the *rag4* mutant. We conclude that the glucose repression of *LAC9* requires Rag4.

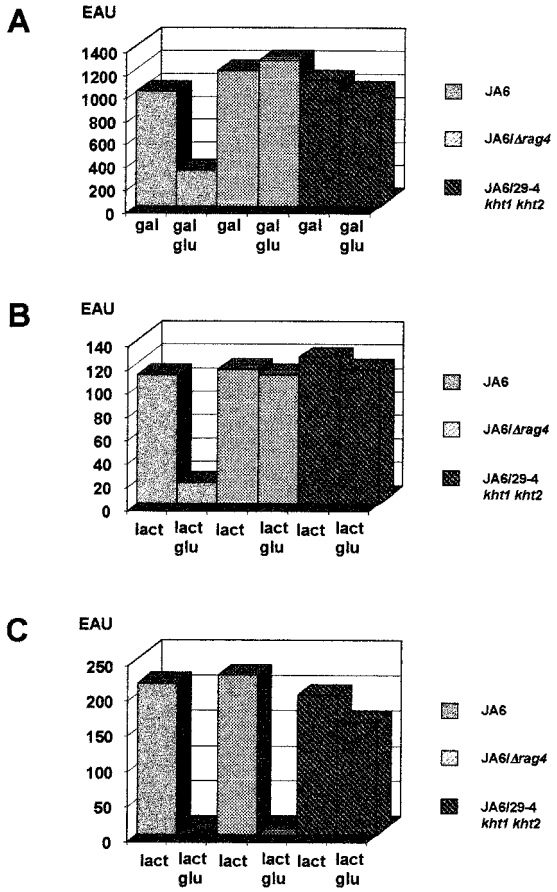

FIGURE 8.—Glucose repression in JA6/Δ*rag4* and JA6/29-4 (*kht1 kht2*) mutants. Cultures were grown in YP medium containing the specific inducers galactose (β-galactosidase) and lactate (L-LCR and maltase), with or without the addition of 2% glucose. (It has been previously demonstrated that in JA6 strain the maximal induction of maltase is observed in lactate; GOFFRINI *et al.* 1995.) (A) β-Galactosidase activity; (B) L-LCR activity; (C) maltase activity. Enzyme activities were measured as described in MATERIALS AND METHODS. Enzyme activity units (EAU) are expressed as nanomoles of substrate per minute (per milligram of protein). All values are means of three independent experiments. In no case was the variation >15%.

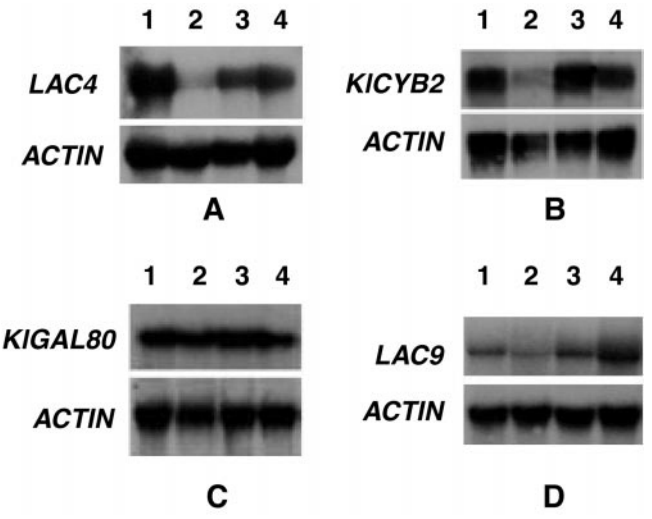

FIGURE 9.—Northern blot analysis of *LAC/GAL* and *KlCYB2* genes in *rag4* null mutant. Total cellular mRNA was prepared from cells grown on 2% galactose (lanes 1 and 3) and 2% galactose plus 2% glucose (lanes 2 and 4). JA6 (*RAG4*) strain (lanes 1 and 2); isogenic JA6/Δ*rag4* strain (lanes 3 and 4). (A) *LAC4* transcription. (B) *KlCYB2* transcription. (C) *KlGAL80* transcription. (D) *LAC9* transcription. The probes used are described in MATERIALS AND METHODS.

DISCUSSION

The *RAG4* gene has been supposed to have some regulatory function in the glucose uptake system in *K. lactis* because, in this mutant, the induced transcription of the glucose transporter gene *RAG1* is lost (CHEN *et al.* 1992; WÉSOLOWSKI-LOUVEL *et al.* 1992b). The transcription of the high-affinity glucose permease gene *HGT1* is also modified in a *rag4* mutant (BILLARD *et al.* 1996). *RAG4* codes for a typical protein of the transmembrane sugar transporter family and shares a high level of identity with two *S. cerevisiae* proteins, Snf3 and Rgt2. Both of these proteins have been proposed to act as glucose sensors that control hexose transporter gene expression in this yeast (ÖZCAN *et al.* 1996, 1998). Like these proteins, Rag4 harbors a characteristic long C-terminal extension that has been shown to be important for glucose signaling function by Snf3 and Rgt2 (COONS *et al.* 1997; ÖZCAN *et al.* 1998; VAGNOLI *et al.* 1998). Also, like the two *S. cerevisiae* genes, *RAG4* was found to be transcribed at a low level, which is consistent with a supposed regulatory function of the product rather than a transport activity.

Snf3 has been proposed to function as a sensor of low levels of glucose and Rgt2 as a sensor of high glucose levels (ÖZCAN *et al.* 1996, 1998). As the *RAG4* gene product regulates positively the low-affinity glucose transporter gene *RAG1* (CHEN *et al.* 1992; this study), one may suppose *a priori* that Rag4 is a high glucose sensor like Rgt2 of *S. cerevisiae*. However, we found that either *SNF3* or *RGT2* can fully complement *in vivo* the *rag4* mutation of *K. lactis*. Therefore, there is the possibility that Rag4 is performing both high and low glucose-sensing functions in *K. lactis*. However, the capacity of Snf3 to complement the *rag4* mutation of *K. lactis* could be due to the ability of the high-affinity glucose sensor to sense high glucose concentrations. To verify whether Rag4 really has a double function, an obvious test is complementation of *snf3* and *rgt2* mutations by *RAG4*. It is unfortunate that the unusual instability of the *RAG4* gene-carrying plasmids in *E. coli* hosts makes this experiment very difficult at this time. The element(s) responsible for this unexplained instability seems to reside within or close to the 5′ end of the *RAG4* gene. Nevertheless, several facts are in favor of the hypothesis that Rag4 has a dual sensing function. First, the growth ability of the *rag4* mutant was altered at both low and high glucose, as it is in the *snf3 rgt2* double mutant of *S. cerevisiae*

(Schmidt *et al.* 1999). Second, high- and low-affinity glucose transport were both affected in a *rag4* mutant. Third, the transcription of the low- and high-affinity glucose transporter genes *RAG1* and *HGT1*, respectively, was impaired or increased in the Δ*rag4* strain of the CBS 2359 genetic background. In the JA6 genetic context where the single gene *RAG1* has been replaced by *KHT1* plus *KHT2*, the transcription of the two genes was no longer induced by high and low levels of glucose, respectively. Fourth, disruption of *RAG4* in a glucose-responsive strain (JA6) abolished glucose repression of several genes (like *LAC4* and *KlCYB2*), whereas in *S. cerevisiae* glucose repression of *SUC2* and *GAL1* can be prevented only in the *snf3 rgt2* double mutant (Özcan *et al.* 1998; Schmidt *et al.* 1999). Finally, we found no other *SNF3/RGT2*-related sequences in *K. lactis* despite our attempts to detect such gene(s) using a probe for the specific 25-codon sequence (the supposed glucose sensor signature) common to the carboxy tail of *RAG4*, *SNF3*, and *RGT2* (Southern hybridization experiments at low stringency; data not shown). Therefore, all data so far available support the idea that *K. lactis* has a single glucose sensor, Rag4, which is able to detect low and high levels of glucose and mediate the signal to glucose transporter genes.

Although less pronounced than in *S. cerevisiae*, glucose repression is also an important regulatory device in some strains of *K. lactis* (JA6 series; Ferrero *et al.* 1978; Breunig 1989; Goffrini *et al.* 1995). When the two glucose permease genes *KHT1* and *KHT2* are deleted, glucose repression of lactose regulon enzymes as well as several other enzymes required for the utilization of lactate, ethanol, maltose, and raffinose (Figure 8) is abolished (Weirich *et al.* 1997). This confirms that glucose uptake is a crucial parameter for glucose repression. The finding that the *rag4* mutation, despite the severe inhibition of *KHT1* and *KHT2* expression, affects glucose repression less than the *kht1 kht2* double mutation could be explained by the presence (though in reduced levels) of Kht1 in the *rag4* mutant, allowing glucose to enter into the cell and to exert repression of some glucose-sensitive genes. It is notable that disruption of the *RAG4* gene in the strain that is not glucose sensitive (MW270-7B) led to a strong reduction of *RAG1* transcription without completely eliminating low-affinity glucose uptake. In *S. cerevisiae* the signal for glucose repression appears to be related to the glucose concentration rather than the glucose flux (Meijer *et al.* 1998), and it seems likely that the intracellular glucose concentration is important (Ye *et al.* 1999). Our present findings, together with previous results establishing that glucose repression is not abolished in a phosphoglucose isomerase mutant (Weirich *et al.* 1997), are in agreement with the results obtained in *S. cerevisiae*. Altogether, these data suggest that glucose needs to enter into the cell but does not need to be metabolized to produce the signal for glucose repression.

We are grateful to Sabira Özcan and Marian Carlson for sharing plasmids. We thank Hiroshi Fukuhara for encouragement and helpful discussions. Svätopluk Betina was a recipient of a Fellowship from the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche. This work was supported in part by a grant from the Commission of the European Communities (BIO4-CT96-0003) and in part by a grant from Ministero Università e Ricerca Scientifica e Tecnologica-Università di Parma Cofin 1999.

LITERATURE CITED

Alberti, A., P. Goffrini, I. Ferrero and T. Lodi, 2000 Cloning and characterization of the lactate-specific inducible gene *KlCYB2*, encoding the cytochrome *b*2 of *Kluyveromyces lacti*. Yeast **16:** 657–665.

Billard, P., S. Ménart, J. Blaisonneau, M. Bolotin-Fukuhara, H. Fukuhara *et al.*, 1996 Glucose uptake in *Kluyveromyces lactis*: Role of the *HGT1* gene in glucose transport. J. Bacteriol **178:** 5860–5866.

Blaisonneau, J., H. Fukuhara and M. Wésolowski-Louvel, 1997 The *Kluyveromyces lactis* equivalent of caseine kinase I is required for the transcription of the gene encoding the low-affinity glucose permease. Mol. Gen. Genet. **253:** 469–477.

Breunig, K. D., 1989 Glucose repression of *LAC* gene expression in yeast is mediated by the transcriptional activator LAC9. Mol. Gen. Genet. **216:** 422–427.

Breunig, K. D., M. Bolotin-Fukuhara, M. M. Bianchi, D. Bourgarel, C. Falcone *et al.*, 2000 Regulation of primary carbon metabolism in *Kluyveromyces lactis*. Enzyme Microb. Technol. **26:** 771–780.

Chen, X. J., 1996 Low- and high-copy-number shuttle vectors for replication in the budding yeast *Kluyveromyces lactis*. Gene **172:** 131–136.

Chen, X. J., M. Wésolowski-Louvel and H. Fukuhara, 1992 Glucose transport in the yeast *Kluyveromyces lactis*. II. Transcriptional regulation of the glucose transporter gene *RAG1*. Mol. Gen. Genet. **33:** 97–105.

Cohn, M., and J. Monod, 1951 Purification et proprieté de la β-galactosidase d'*Escherichia coli*. Biochem. Biophys. Acta **7:** 153–165.

Coons, D., M. P. Vagnoli and L. F. Bisson, 1997 The C-terminal domain of Snf3p is sufficient to complement the growth defect of *snf3* null mutations in *Saccharomyces cerevisiae*: *SNF3* functions in glucose recognition. Yeast **13:** 9–20.

Dickson, R. C., R. M. Sheetz and L. R. Lacy, 1981 Genetic regulation: yeast mutants constitutive for β-galactosidase activity have an increased level of β-galactosidase messenger ribonucleic acid. Mol. Cell. Biol. **1:** 1048–1056.

Dohmen, R. J., A. W. M. Strasser, C. B. Höner and C. P. Hollenberg, 1991 An efficient transformation procedure enabling long-term storage of competent cells of various yeast genera. Yeast **7:** 691–692.

Ferrero, I., C. Rossi, M. P. Landini and P. P. Puglisi, 1978 Role of the mitochondrial protein synthesis in the carbon catabolite repression of the petite-negative yeast *K. lactis*. Biochem. Biophys. Res. Commun. **80:** 340–348.

Goffrini, P., A. A. Algeri, C. Donnini, M. Wésolowski-Louvel and I. Ferrero, 1989 *RAG1* and *RAG2*: nuclear genes involved in the dependence/independence on mitochondrial respiratory function for the growth on sugars. Yeast **5:** 99–106.

Goffrini, P., A. Ficarelli and I. Ferrero, 1995 Hexokinase activity is affected in mutants of *Kluyveromyces lactis* resistant to glucose repression. Microbiology **141:** 441–447.

Liang, H., and R. F. Gaber, 1996 A novel signal transduction pathway in *Saccharomyces cerevisiae* defined by Snf3-regulated expression of *HXT6*. Mol. Biol. Cell **7:** 1953–1966.

Lodi, T., D. O'Connor, P. Goffrini and I. Ferrero, 1994 Isolation and characterization of the *KlDLD* gene encoding the carbon catabolite repressible mitochondrial enzyme D-lactate ferricytochrome c oxidoreductase in *Kluyveromyces lactis*. Mol. Gen. Genet. **244:** 622–629.

Marshall-Carlson, L., J. L. Celenza, B. C. Laurent and M. Carl-

son, 1990 Mutational analysis of the SNF3 glucose transporter of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 1105–1115.

Meijer, M., M. C. J. Boonstra, A. J. Verkleij and C. T. Verrips, 1998 Glucose repression in *Saccharomyces cerevisiae* is related to the glucose concentration rather than the glucose flux. J. Biol. Chem. **273:** 24102–24107.

Neigeborn, L., P. Schwartzberg, R. Reid and M. Carlson, 1986 Null mutation in the *SNF3* gene of *Saccharomyces cerevisiae* cause a different phenotype than do previously isolated missense mutations. Mol. Cell. Biol. **6:** 3569–3574.

Özcan, S., J. Dover, A. G. Rosenwald, S. Wölfl and M. Johnston, 1996 Two glucose transporters in *Saccharomyces cerevisiae* are glucose sensors that generate a signal for induction of gene expression. Proc. Natl. Acad. Sci. USA **93:** 12428–12432.

Özcan, S., J. Dover and M. Johnston, 1998 Glucose sensing and signaling by two glucose receptors in the yeast *Saccharomyces cerevisiae*. EMBO. J. **17:** 2566–2573.

Prior, C. P., Mamessier, H. Fukuhara, X. J. Chen and M. Wésolowski-Louvel, 1993 The hexokinase gene is required for the transcriptional regulation of the glucose transporter gene *RAG1* in *Kluyveromyces lactis*. Mol. Cell. Biol. **13:** 3882–3889.

Salmeron, J., M., and S. A. Johnston, 1986 Analysis of the *Kluyveromyces lactis* positive regulatory gene *LAC9* reveals functional homology to, but sequence divergence from, the *Saccharomyces cerevisiae GAL4* gene. Nucleic Acids Res. **14:** 7767–7781.

Schmidt, M., C. R. R. McCartney, X. Zhang, T. S. Tillman, H. Solimeo *et al.*, 1999 Std1 and Mth1 proteins interact with the glucose sensors to control glucose-regulated gene expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **19:** 4561–4571.

Thierry, A., C. Fairhead and B. Dujon, 1990 The complete sequence of the 8.2 kb segment left of MAT on chromosome III reveals five ORFs, including a gene for a yeast ribokinase. Yeast **6:** 521–534.

Vagnoli, P., D. M. Coons and L. F. Bisson, 1998 The C-terminal domain of Snf3p mediates glucose-responsive signal transduction in *Saccharomyces cerevisiae*. FEMS Microbiol. Lett. **160:** 31–36.

Weirich, J., P. Goffrini, P. Kuger, I. Ferrero and K. D. Breunig, 1997 Influence of mutations in hexose-transporter genes on glucose repression in *Kluyveromyces lactis*. Eur. J. Biochem. **249:** 248–257.

Wésolowski, M., A. A. Algeri, P. Goffrini and H. Fukuhara, 1982 Killer DNA plasmids of the yeast *Kluyveromyces lactis*. I. Mutations affecting the killer phenotype. Curr. Genet. **150:** 137–140.

Wésolowski-Louvel, M., P. Goffrini, I. Ferrero and H. Fukuhara, 1992a Glucose transport in the yeast *Kluyveromyces lactis*. I. Properties of an inducible low-affinity glucose transporter gene. Mol. Gen. Genet. **33:** 89–96.

Wésolowski-Louvel, M., C. Prior, D. Bornecque and H. Fukuhara, 1992b Rag$^-$ mutations involved in glucose metabolism in yeast: Isolation and genetic characterization. Yeast **8:** 711–719.

Ye, L., A. L. Kruckeberg, J. A. Berden and K. Van Dam, 1999 Growth and glucose repression are controlled by glucose transport in *Saccharomyces cerevisiae* cells containing only one glucose transporter. J. Bacteriol. **181:** 4673–4675.

Zenke, F., W. Zachariae, A. Lunkes and K. D. Breunig, 1993 Gal80 proteins of *Kluyveromyces lactis* and *Saccharomyces cerevisiae* are highly conserved but contribute differently to glucose repression of the galactose regulon. Mol. Cell. Biol. **13:** 7566–7576.

Zimmermann, F. K., and I. Scheel, 1977 Mutants of *Saccharomyces cerevisiae* resistant to carbon catabolite repression. Mol. Gen. Genet. **154:** 75–82.

Zimmermann, F. K., I. Kaufmann, H. Rasenberger and P. Hausmann, 1977 Genetics of carbon catabolite repression in *Saccharomyces cerevisiae*: genes involved in the derepression process. Mol. Gen. Genet. **151:** 95–103.

Communicating editor: M. Johnston