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Construction of strains of *Saccharomyces cerevisiae* that grow on lactose

(recombinant DNA/yeasts/*Kluyveromyces lactis*/lactose permease/whey)

K. SREEKRISHNA* AND ROBERT C. DICKSON†

Department of Biochemistry, University of Kentucky, Lexington, KY 40536-0084

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ABSTRACT **We have constructed strains of *Saccharomyces cerevisiae* that grow on lactose (Lac$^+$). *S. cerevisiae* strain YNN27, which, like all *S. cerevisiae*, is unable to grow on lactose, was transformed with pKR1B-LAC4-1. This plasmid has a selectable marker gene conferring resistance to the antibiotic G418 and carries a 13-kilobase region of the *Kluyveromyces lactis* genome including *LAC4*, a β-galactosidase gene. Transformants were selected first for G418 resistance and then for growth on lactose. Southern hybridization experiments showed that Lac$^+$ transformants had integrated 15–25 tandem copies of the vector into a host chromosome. Several lines of evidence indicate that the Lac$^+$ phenotype in pKR1B-LAC4-1-transformed *S. cerevisiae* is due to expression of a *K. lactis* lactose permease gene that lies between 2 and 8.6 kilobases upstream of *LAC4* and also to expression of *LAC4*. The permease gene has been designated *LAC12*.**

The lactose in whey, the by-product of cheese making, represents a potential substrate for the production of ethanol and the growth of microorganisms. However, this potential has not been realized on a large, economically feasible scale. As a result, a great deal of whey must be disposed of. Disposal of the lactose in whey is a problem because it requires costly waste treatment processes. Attempts to use the lactose in whey have included fermentation by strains of *Kluyveromyces fragilis* (1–3) and other yeasts, especially *Saccharomyces cerevisiae* (4). The major difficulty with *S. cerevisiae* is that it cannot ferment lactose directly; the lactose must be hydrolyzed before the resultant glucose and galactose can be utilized. Such a procedure is hampered by high concentrations of extracellular glucose, which cause catabolite repression of galactose utilization. Catabolite repression can be partially overcome by selecting mutant strains that are resistant to repression (4). Despite these difficulties, *S. cerevisiae* and related species are prime candidates for attempting to ferment lactose because they have long been used by the brewing industry and procedures for their use in commercial-scale production are highly developed. Another reason for attempting to use *S. cerevisiae* is that it can be manipulated genetically by numerous techniques including genetic engineering (5). Also, it would be highly advantageous to have strains of *S. cerevisiae* that could grow on lactose because they could be used in a variety of mutant-selection schemes, as has been done with *Escherichia coli* (6).

S. cerevisiae cannot utilize lactose because it does not have a β-galactosidase structural gene and therefore cannot hydrolyze lactose to glucose and galactose and because it has no mechanism for transporting lactose across the cell membrane. This problem has been demonstrated by direct measurement of lactose transport and by showing that genetically engineered strains of *S. cerevisiae* that make intracellular β-galactosidase do not grow on lactose (7). One solution to the transport problem may be to introduce a lactose permease gene, such as the *lacY* gene of *E. coli*, into a strain of *S. cerevisiae* that makes β-galactosidase. This approach has not proven successful (unpublished results). An alternative approach would be to use a lactose permease gene from another yeast.

The yeast *Kluyveromyces lactis* can grow on lactose as a sole carbon source and has an inducible lactose permease system (8). The gene(s) coding for the permease has not been identified until now. In this paper, we present the results of experiments in which we transformed *S. cerevisiae* with a plasmid carrying the β-galactosidase gene (*LAC4*) and flanking sequences from *K. lactis*, as well as the kanamycin-resistance gene from *E. coli*, which confers resistance to G418 in yeast. We selected transformants first for resistance to the antibiotic G418 and then for growth on lactose. We found that a DNA fragment located 2 kilobases upstream from *LAC4* can be used to construct strains of *S. cerevisiae* that transport lactose. If a β-galactosidase gene, either the *lacZ* of *E. coli* or the *LAC4* of *K. lactis*, is also present, the strains grow on lactose. To our knowledge, this is the first time that a eukaryotic membrane-bound permease has been expressed in a heterologous organism.

MATERIALS AND METHODS

Bacterial and Yeast Strains. *E. coli* strain DG75 (*hsd1 leu6 ara14 galK2 xyl5 mtl1 rpsL20 thi1 supE44 lac*ΔZ39 λ$^-$) was used for all bacterial transformations and plasmid propagations. *S. cerevisiae* strains YNN27 (α *trp1-289 ura3-52 gal2*; ref. 9) and L1582 (α *ino HIS4::lacZ*) were obtained from G. R. Fink (Massachusetts Institute of Technology, Cambridge, MA). *K. lactis* wild-type strain Y1140 (a *lac1 LAC2*) and mutant strain MS425 (α *lac4-8 ade1-1*) have been described (10).

Chemicals and Enzymes. G418 sulfate was from GIBCO, and kanamycin and ampicillin were from Sigma. Restriction enzymes, enzyme grade bovine serum albumin, and DNA polymerase I (Klenow fragment) were products of Bethesda Research Laboratories. [D-*glucose*-1-^{14}C]lactose was from Amersham (58 mCi/mmol; 1 Ci = 37 GBq).

Media. YPD medium contained 20 g of glucose, 20 g of peptone, and 10 g of yeast extract per liter; YP medium lacked dextrose. Minimal lactose medium (MinLac) contained 3.4 g of yeast nitrogen base (Difco, without amino acids or ammonium sulfate), 10 g of ammonium sulfate, 20 g of lactose, 10 mg of adenine, 10 mg of uracil, 10 mg of leucine, 10 mg of tryptophan, 10 mg of histidine, 10 mg of methionine, and 10 mg of lysine per liter.

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Abbreviations: MinLac, minimal lactose medium; YPD medium, yeast extract/peptone/dextrose medium; G418^R, G418 resistant.

*Present address: Phillips Petroleum Co., Bartlesville, OK 74004.
†To whom reprint requests should be addressed.

Plasmids. Plasmid pKR1B has been described (ref. 11; Fig. 1). The other plasmids described in Fig. 1 are from Bhairi (12).

Measurement of Lactose Transport. The procedure for measuring lactose transport was that described by Serrano (13), except for minor changes. Lac$^-$ strains of *S. cerevisiae* transformed with any of the plasmids shown in Fig. 1 were grown at 30°C to saturation in YP medium containing 2% (wt/vol) lactic acid (titrated to pH 4.2 before addition to YP), 2% glycerol, and G418 at 200 μg/ml. *K. lactis* strains were grown in the same medium except that G418 was used at 10 μg/ml and galactose was added to 1% (wt/vol). Lac$^+$ *S. cerevisiae* was grown in MinLac (1% or 2% lactose) medium. This medium selects for Lac$^+$ cells, which is particularly important when dealing with unstable transformants. It should be noted that G418 selection cannot be conducted in minimal media (11). The specific activity, cpm/pmol of lactose, in each reaction mixture was determined from the total cpm in a 10-μl sample. Data are expressed as pmol of intracellular lactose per OD_{600} unit. Background transport was measured using cells that were heated for 3 min at 90°C. Typical background was 25–30 cpm.

Miscellaneous Procedures. Procedures for transforming *E. coli*, preparing plasmid DNA from *E. coli* and total DNA from yeasts, and Southern hybridizations have been described (11). *S. cerevisiae* was transformed according to Hinnen *et al.* (14) and G418-resistant transformants were selected as described (11, 15). Plasmid copy number was determined by probing Southern blots with [^{32}P]oligonucleotide-labeled

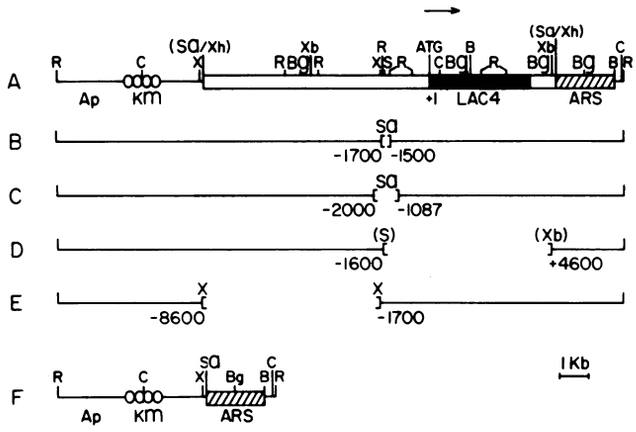

FIG. 1. Structure of yeast plasmids. Construction of the yeast shuttle vector pKR1B (line F) has been described (12). This plasmid carries genes conferring resistance to ampicillin (Ap) and kanamycin (Km) in *E. coli*, plus the pBR322 origin of DNA replication that allows replication in *E. coli* and a *K. lactis* autonomous replication sequence (*ARS*) that allows replication in both *K. lactis* and *S. cerevisiae*. In yeast the kanamycin-resistance gene confers resistance to the antibiotic G418. Construction of pKR1B-LAC4-1 (line A) and derivatives of it carrying deletions (lines B–E) have been described (12). *LAC4* is a β-galactosidase structural gene (10). Its direction of transcription is shown by an arrow above the diagram, and its ATG initiation codon is indicated (12). Numbers below the plasmid sequences represent nucleotide distances relative to adenosine +1 in the initiation codon. All distances were determined from the lengths of the restriction fragments, except position −1087, which was determined from DNA sequencing (12). DNA sequences are diagrammed in lines A and F as follows: solid thin lines, *E. coli* pBR322; circles, Tn*903* sequences; open bars, *K. lactis* sequences; solid bar, *LAC4* of *K. lactis*; hatched bars, *K. lactis ARS1B*. Restriction endonuclease sites are as follows: R, *Eco*RI, B, *Bam*HI; C, *Cla* I; Sa, *Sal* I; Xh, *Xho* I; Xb, *Xba* I; Bg, *Bgl* II; S, *Sst* II; X, *Xma* III. Plasmids shown in lines B–E have the same DNA sequences as pKR1B-LAC4-1 (line A), except that they lack the bracketed sequences. Restriction sites shown in parentheses were inactivated during plasmid construction.

YRp7 cleaved with *Eco*RI. This plasmid has a 1.45-kilobase *S. cerevisiae* fragment, containing the *TRP1* and *ARS1* genes, cloned at the pBR322 *Eco*RI site. Autoradiograms were quantitated by densitometry and sequences hybridizing to the pBR322 portion of the probe were normalized to the two small *Bam*HI/*Bgl* II fragments hybridizing to the *TRP1*/*ARS1* portion of the probe, which should be present at one copy each per haploid cell.

RESULTS

Construction of *S. cerevisiae* Strains That Grow on Lactose. After unsuccessful direct attempts to select *S. cerevisiae* capable of growth on lactose following transformation with recombinant plasmids, we undertook a two-step procedure using a plasmid that carries both β-galactosidase and antibiotic-resistance genes and selecting first for antibiotic resistance and then for growth on lactose. This approach gave *S. cerevisiae* that grew on lactose. *S. cerevisiae* strain YNN27 was transformed with pKR1B-LAC4-1 (Fig. 1, line A) and G418-resistant (G418^R) colonies were selected. Strain YNN27 was used because it transforms at high frequency, and we have used it previously for selecting G418^R yeast transformants (15). Plasmid KR1B-LAC4-1 was used for reasons presented in the *Discussion*.

In one experiment, three G418^R colonies were streak-purified on YPD/G418 plates, and a single colony from each of the three isolates was pooled. The pooled cells were plated at various concentrations on YPD, YPD/G418, and MinLac plates. After 4–6 days of incubation at 30°C, Lac$^+$ colonies arose on the MinLac plates. Their frequency was 1 per 250 G418^R colonies or 1 per 1000 viable cells (only 25% of the viable cells were G418^R). The frequency of G418^R cells and Lac$^+$ cells varied from one experiment to another. For example, in another transformation experiment, Lac$^+$ cells arose at a frequency of 1 per 7 G418^R colonies or 1 per 175 viable cells. The difference in frequencies may be due to the selection procedure used. In the first experiment, cells were grown on YPD plates containing G418 at 200 mg/ml prior to selection on MinLac plates while, in the second experiment, cells were grown in liquid medium (YP plus 2% lactic acid and 2% glycerol containing G418 at 100 mg/ml) prior to selection on MinLac plates. The low frequency of G418^R colonies even under growth conditions that select for G418 resistance is probably due to the autonomous replication sequence (*ARS*) present on the vector. We (11, 15) and others (9, 16) have noted that yeast vectors containing *ARS* replicons are not segregated to every daughter cell at mitosis. Consequently, many G418-sensitive cells exist in a population.

In control transformation experiments Lac$^+$ cells were never obtained when YNN27 was transformed with pKR1B (Fig. 1, line F), the parent to pKR1B-LAC4-1.

Lac$^+$ *S. cerevisiae* Has Integrated pKR1B-LAC4-1 into a Chromosome. To show that the Lac$^+$ phenotype of *S. cerevisiae* is due to the presence of pKR1B-LAC4-1 and to determine whether the plasmid was replicating autonomously or had integrated into a host chromosome, we used Southern hybridization analysis (Fig. 2). Total DNA isolated from six independent Lac$^+$ G418^R transformants of strain YNN27 showed one band of hybridization corresponding to chromosomal DNA when the Southern blot was probed with ^{32}P-labeled pKR1B-LAC4-1 (one example is shown in lane B). If the vector had been present in the autonomous or unintegrated state we should have seen two bands hybridizing to the probe, the faster migrating band being supercoiled and the other being open-circular vector DNA (lane A).

Further evidence for vector integration was obtained by using *Bam*HI/*Bgl* II-cleaved total yeast DNA. If one copy of the plasmid had integrated we would expect one of the five *Bam*HI–*Bgl* II vector DNA fragments (bands, lane C) to be

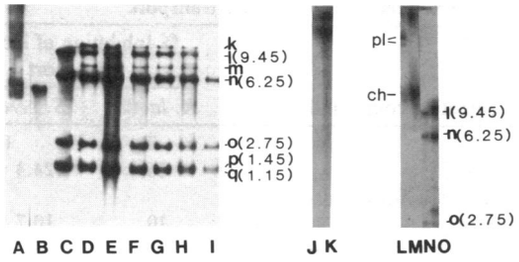

FIG. 2. Southern hybridization analysis of pKR1B-LAC4-1 sequences in *S. cerevisiae*. Total DNA was extracted from yeast, electrophoresed on 0.9% agarose gels, and hybridized to *Bam*HI/*Bgl* II-cleaved ^{32}P-labeled pKR1B-LAC4-1 by the procedure of Southern as described previously (11, 15). Numbers in parentheses represent lengths in kilobase pairs [determined by comparison with phage λ DNA molecular weight standards (not shown)]. Lanes: A, uncleaved purified pKR1B-LAC4-1; B, uncleaved Lac$^+$ YNN27/pKR1B-LAC4-1; C, purified *Bam*HI/*Bgl* II-cleaved pKR1B-LAC4-1; D–I, *Bam*HI/*Bgl* II-cleaved Lac$^+$ YNN27/pKR1B-LAC4-1; J, uncleaved YNN27; K, uncleaved YNN27/pKR1B-LAC4-1; L and M, uncleaved G418^R Lac$^-$ YNN27/pKR1B-LAC4-1; N and O, *Bam*HI/*Bgl* II-cleaved G418^R Lac$^-$ YNN27/pKR1B-LAC4-1. The gel for lanes J–O contained ethidium bromide (1 μg/ml) during electrophoresis, which resulted in clearer separation of chromosomal and autonomous vector DNAs than was obtained with gels lacking ethidium bromide (lanes A and B).

absent from the Southern blot and two new bands representing chromosomal sequences flanking the integrated vector to be present. These bands could be any size. If more than one copy of the vector had integrated tandemly, as often happens (14, 15), all five vector bands plus the two new bands should be observed. Five of the DNA samples (lanes D–G and I) gave five bands corresponding to plasmid DNA fragments plus two new less intense bands (bands k and m). Thus, these five Lac$^+$ G418^R transformants contained multiple tandem copies of pKR1B-LAC4-1. Integration of the vector occurred at the same chromosomal locus in all five transformants since bands k and m are the same size. The transformant whose DNA is present in lane H seems to have integrated the vector at two sites since there is a band, just above band m, that most probably is due to a new flanking sequence although it could also be due to deletion of vector sequences. All six of these transformants carried 15–25 copies of the vector per cell based on densitometric scans of the autoradiograms (data not shown). In control experiments, ^{32}P-labeled pKR1B-LAC4-1 did not hybridize to DNA from untransformed YNN27 (lane J; compare with lane K).

These data suggest that the Lac$^+$ phenotype arises only when pKR1B-LAC4-1 integrates into one or a few specific chromosomal locations. Further support for this hypothesis was obtained by examining vector sequences in transformants selected for G418 resistance only. Their phenotypes are G418^R Lac$^-$. We first determined that all vector sequences were present, since the Lac$^-$ phenotype might be due to mutations in the vector. Total DNA from 20 transformants was analyzed by Southern blotting using either uncleaved or *Bam*HI/*Bgl* II-cleaved DNA. Thirteen of the 20 samples contained unintegrated, autonomous vectors, while the other 7 contained integrated vector sequences. One representative of a strain carrying integrated vector sequences is shown in Fig. 2, lane M. There are two bands of hybridizing DNA. The upper band is due to high molecular weight aggregates of chromosomal DNA that barely enter the gel, while the lower band is due to uncleaved chromosomal DNA (ch). Lane L shows a representative with autonomous vector sequences that appear as two distinct bands below the sample well and are labeled pl. This representative may also have some vector sequences that have integrated into a chromosome since there is a faint band that migrates midway down the lane in a position corresponding to uncleaved chromosomal DNA. The lowest, faint band in the lane was also present in purified vector DNA and may be due to a minor deleted species of pKR1B-LAC4-1 that replicates autonomously. We predicted that the G418^R Lac$^-$ transformant with integrated vector sequences would show at least two new bands, due to sequences flanking the integration site, when total DNA was digested with *Bam*HI and *Bgl* II and analyzed by Southern blotting. This prediction was verified, as shown in lane O, in which there are two bands (the upper one and the second from the bottom) that were not present in the G418^R Lac$^-$ transformant having autonomous vector sequences (lane N). The gel used for lanes N and O was electrophoresed for an extended period of time so that the two fastest migrating vector sequences, bands p and q, are not seen. Their presence was verified in a separate Southern blot (data not shown). Thus, by the criterion of DNA fragment length, these two types of G418^R Lac$^-$ transformants contain all of the vector sequences and should be capable of yielding a Lac$^+$ phenotype. This prediction was verified by selection of Lac$^+$ transformants from all 20 G418^R Lac$^-$ strains by plating 10^4–10^6 cells on MinLac plates. These results show that the mere presence of pKR1B-LAC4-1 in YNN27 in either the autonomous or the integrated state does not yield a Lac$^+$ phenotype. The flanking sequences in the Lac$^-$ integrant (lane O) are different from those in the Lac$^+$ integrants (lanes D–I, bands k and m), indicating that integration must occur at one or perhaps a few loci for the host to become Lac$^+$. The transformant shown in lane O had the same number of integrated vector sequences as the transformants shown in lanes D–I, indicating that the number of copies of the vector does not account for the difference in Lac phenotype between these transformants.

Which Region of pKR1B-LAC4-1 Confers the Lac$^+$ Phenotype on *S. cerevisiae*? We assumed at this point that pKR1B-LAC4-1 was conferring the Lac$^+$ phenotype on *S. cerevisiae* because the plasmid had, in addition to *LAC4*, a gene coding for a lactose permease. To determine which region of pKR1B-LAC4-1 coded for the presumptive permease, *S. cerevisiae* strain L1582 was transformed with a set of deletion plasmids (Fig. 1, plasmids shown in lines B–E), G418^R transformants were selected and subsequently plated on MinLac plates at 10^4 and 10^6 cells per plate. Strain L1582 has a portion of the *E. coli lac* operon fused to the yeast *HIS4* gene, *HIS4*::*lacZ*, and thus makes β-galactosidase. We reasoned that this strain would become Lac$^+$ when transformed with derivatives of pKR1B-LAC4-1 that contained the permease gene; there would be no need for *LAC4*. Lac$^+$ colonies were obtained with the plasmids shown in Fig. 1, lines B–D, but none were obtained with that shown in line E. We conclude from these data that a region of pKR1B-LAC4-1 (Fig. 1*A*) between −2000 and −8600 conveys the Lac$^+$ phenotype on strain L1582 and thus codes for a lactose permease gene, which we designate *LAC12*.

Characterization of Lactose Transport in Lac$^+$ *S. cerevisiae*. The most likely reason why pKR1B-LAC4-1 confers a Lac$^+$ phenotype on *S. cerevisiae* is that it codes for a lactose permease. If this explanation is correct we would expect lactose transport in Lac$^+$ *S. cerevisiae* and *K. lactis* to have similar properties. Initially the kinetics of lactose transport were measured to determine whether *Lac*$^+$ *S. cerevisiae* transported measurable quantities of lactose and to determine the time interval over which the apparent initial velocity of transport could be measured. Lactose (0.5 mM) uptake was linear for at least 3 min and, during this interval, Lac$^+$ *S. cerevisiae* strains L1582/pKR1B-LAC4-Δ1087 and YNN-27/pKR1B-LAC4-1 and *K. lactis* strain MS425/pKR1B transported 85, 40, and 205 pmol of lactose per OD_{600} unit, respectively. Lac$^-$ L1582 transformed with the parent vector

pKR1B did not transport lactose even during a 20-min incubation period.

If lactose is being transported by a membrane-bound permease it should be possible to demonstrate saturation of the transport process at high concentrations of lactose. Alternatively, if lactose is simply diffusing across the membrane then substrate saturation should not occur. As shown in Fig. 3, lactose transport in Lac$^+$ strains becomes saturated with higher concentrations of lactose. Lineweaver–Burk plots of these data gave apparent K_m values of 0.97 and 1.09 mM (two determinations) for lactose transport. *K. lactis* strain MS425 had an apparent K_m of 0.67 ± 0.11 (four determinations) for lactose transport using identical procedures. We conclude that lactose transport in Lac$^+$ YNN27/pKR1B-LAC4-1 is a carrier-mediated process (a permease) that has an apparent K_m that is similar to the K_m for lactose transport in *K. lactis*.

Carrier-mediated membrane transport processes generally display a high degree of stereospecificity for substrate. If the *K. lactis* lactose permease gene is responsible for the Lac$^+$ phenotype of strain YNN27/pKR1B-LAC4-1, lactose transport in this strain should show the same stereospecificity for substrate as *K. lactis* (8). Stereospecificity was shown by measuring how well a compound inhibited lactose transport (Table 1). Since lactose, 4-*O*-β-D-galactosyl-D-glucose, is a β-galactoside we would expect the α-galactosides, melibiose and 4-nitrophenyl-α-D-galactose, to inhibit lactose transport only weakly and they did (Table 1). The lactose permease shows specificity for a disaccharide because the monosaccharide galactose gave only moderate inhibition. The permease also preferred an *O*-linkage to a thio-linkage since thiodigalactoside gave only moderate inhibition. The strongest inhibition was shown by a disaccharide with a β-*O*-linkage, 3-*O*-β-D-galactosyl-D-arabinose.

Lastly we examined whether lactose transport in Lac$^+$ *S. cerevisiae* is an energy-dependent process, as it is in *K. lactis* (8). To measure energy dependence, cells were preincubated for 20 min with 1 mM 2,4-dinitrophenol at 23°C. Lactose transport was inhibited 91% in both the Lac$^+$ *S. cerevisiae* strain YNN27/pKR1B-LAC4-1 and *K. lactis* strain MS425/pKR1B, implying a similar energy-dependent transport mechanism in these two yeasts.

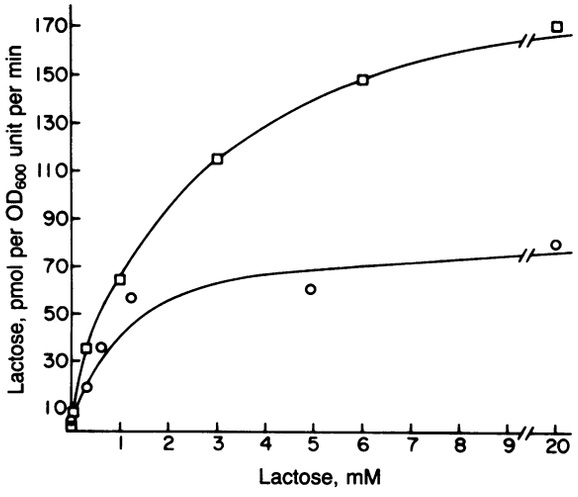

FIG. 3. Lactose transport in Lac$^+$ *S. cerevisiae* is carrier-mediated. The rate of lactose transport as a function of substrate concentration was determined using the Lac$^+$ strains YNN27/pKR1B-LAC4-1 (○) and YNN27/pKR1B-LAC4-Δ1087 (□). For each concentration of lactose, the rate of transport shown represents the average of samples taken at 30, 60, and 90 sec.

Table 1. Stereospecificity of lactose transport

Competing compound	% Inhibition of lactose transport	
	K. lactis	*S. cerevisiae*
None	0	0
D-Galactose	26	24.3 ± 7.2
6-*O*-α-D-galactosyl-D-glucose (melibiose)	10	10.7 ± 8
3-*O*-β-D-galactosyl-D-arabinose	93	90 ± 6
4-Nitrophenyl α-D-galactoside	6	1.1 ± 1
D-galactosyl 1-thio-β-D-galactoside (thiodigalactoside)	28	4.9 ± 4

After preparation for lactose transport measurements, cells (*K. lactis*, MS425/pKR1B; *S. cerevisiae*, YNN27/pKR1B-LAC4-1) were warmed to 23°C and competing compounds were added to a final concentration of 50 mM. Thirty seconds later, [^{14}C]lactose was added to a final concentration of 1 mM and 100-μl samples were filtered 1 and 2 min later. The concentration of inhibitor was chosen so that, if it had the same K_m for transport as lactose (1 mM used for the calculation), lactose transport would be inhibited by 97% [$50K_m/(50K_m + K_m + 0.25K_m)$ = % inhibition]. For *K. lactis* the experiment was done twice and for *S. cerevisiae* it was done three times.

Growth Rate and Stability of Lac$^+$ *S. cerevisiae*. The growth rate of Lac$^+$ *S. cerevisiae* was determined by measuring the doubling time of cells in MinLac medium at 30°C. Doubling times were as follows: *K. lactis* wild-type strain Y1140 = 1.6 hr; *S. cerevisiae* strain YNN27/pKR1B-LAC4-1 = 6.7 hr.

The stability of the Lac$^+$ and G418^R phenotypes in YNN27/pKR1B-LAC4-1 strains was measured by growing cells for 20 generations under nonselective conditions in YPD medium. After growth cells were plated onto YPD and then replicated to MinLac and YPD/G418 plates. Ten strains were examined and they all behaved similarly. Ninety-seven percent of the cells remained G418 resistant but only 18% were Lac$^+$. Southern blotting of DNA from two G418^R Lac$^-$ isolates showed that the *K. lactis* sequences in the vector were no longer present and that the pBR322 and Km gene sequences were still present (data not shown). Therefore the Lac$^-$ phenotype seems to have arisen by deletion of most of the *K. lactis* sequences from the integrated vectors.

DISCUSSION

We have presented evidence that the plasmid pKR1B-LAC4-1 confers a Lac$^+$ phenotype on two different strains of *S. cerevisiae*. The Lac$^+$ phenotype is most reasonably explained by the presence of a *K. lactis* lactose permease gene on pKR1B-LAC4-1. Our data support this hypothesis. First, as in *K. lactis*, lactose transport in Lac$^+$ *S. cerevisiae* is mediated by a transport system that is saturable by lactose (Fig. 3) and has an apparent K_m for transport of about 1 mM. While these data do not prove that the two lactose transport systems are identical, they clearly rule out the possibility that lactose is passively diffusing into *S. cerevisiae* because of a nonspecific membrane dysfunction due to pKR1B-LAC4-1. Second, lactose transport in Lac$^+$ *S. cerevisiae* and *K. lactis* is inhibited to the same extent by 2,4-dinitrophenol, implying that in both organisms the transport system is driven by a membrane potential. This rules out the possibility that lactose transport in Lac$^+$ *S. cerevisiae* occurs by facilitated (carrier-mediated) diffusion. Third, Lac$^+$ *S. cerevisiae* shows stereospecific transport of lactose and the specificity is the same as that shown for *K. lactis* (Table 1). Finally, we localized the permease gene, which we designate *LAC12*, to a region between 2 and 8.6 kilobases upstream of the 5′ end of *LAC4*.

Our finding that the β-galactosidase and lactose permease genes are closely linked was fortuitous. We were encouraged

to look for some linkage by the work of Needleman *et al.* (17), who showed close linkage of a maltase (α-D-glucosidase, EC 3.2.1.20) and a maltase permease gene at the *MAL6* locus of *S. cerevisiae*. Plasmid KR1B-LAC4-1 was used for these studies because it contains one of the largest *K. lactis* DNA inserts that has a functional β-galactosidase gene (12) and might therefore also contain a linked permease gene.

All of our data suggest that the Lac^+ phenotype occurs in YNN27 only when pKR1B-LAC4-1 integrates into a host chromosome and only when it integrates at certain loci. Integration seems to be necessary to express the permease gene and not the β-galactosidase gene since strain L1582, which has high levels of β-galactosidase activity and therefore does not need to express the *LAC4* region of pKR1B-LAC4-1, nevertheless, must integrate the vector to become Lac^+ (data not shown). Vector integration most likely serves to activate transcription of the permease gene, either by fusing it to a host promoter or by separating it from linked *K. lactis* sequences that prevent transcription or translation.

The Lac^+ phenotype does not seem to result from increased gene dosage since Lac^+ and Lac^- YNN27 transformants contain equal numbers of integrated pKR1B-LAC4-1 (15–25 copies). We do not know whether the elevated copy number is due to the G418 or lactose selection pressure or is simply a result of the integration process.

It is not apparent why the $G418^R$ phenotype is stable in YNN27/pKR1B-LAC4-1 while the Lac^+ phenotype is unstable. The *K. lactis* region of pKR1B-LAC4-1 may contain sequences that undergo recombination at a high frequency or that, in some unknown way, interfere with cell growth and thus put their host at a selective disadvantage. Because the *K. lactis ARS* on pKR1B-LAC4-1 functions in *S. cerevisiae*, it probably has DNA sequences that are at least partially homologous to *S. cerevisiae* sequences, so that the *ARS* could undergo homologous recombination with host sequences and be deleted.

The most important experimental element that led to the selection of Lac^+ *S. cerevisiae* was the use of indirect selection. Lac^+ *S. cerevisiae* was never isolated when YNN27 or L1582 was transformed with pKR1B-LAC4-1 and selected directly on MinLac plates; Lac^+ transformants were obtained only when selected first on G418 plates and then on lactose plates. Others have noted a similar phenomenon. For example, the *GAL4* gene of *S. cerevisiae* could not be selected directly for complementation of a *gal4* strain on minimal galactose plates; the gene could be selected only indirectly (18, 19). It is possible that vector-borne genes entering a spheroplasted cell are not expressed well enough initially to meet the huge demand for carbon and energy, so that the cell dies. Indirect selection, on the other hand, places less-stringent demands on the cell because the initial selection pressure can be met by the vector-borne selectable marker gene. During subsequent cell growth and division, the nonselected vector-borne genes have time to reach a steady-state level of expression that is high enough to allow selection for carbon source utilization. The steady-state level of expression may be due to one or more factors, including increased copies of the vector per cell and an increased rate of transcription if the vector-borne genes are regulated.

We believe that the results presented in this paper will have wide usage in both commercial applications and basic research.

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