Construction of strains of Saccharomyces cerevisiae that grow on lactose
(recombinant DNA/yeasts/Kluyveromyces lactis/lactose permease/whey)

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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 LAC2.

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 lacT 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 (hslI leu6 araI4 galK2 xylI mtlI rpsL20 thlI supE44 lacA39 ΔlAr -) was used for all bacterial transformations and plasmid propagations. S. cerevisiae strains YNN27 (a trpl-289 ura3-52 gal2 [ref. 9] and L1582 (a ino HIS4: lacZ) were obtained from G. R. Fink (Massachusetts Institute of Technology, Cambridge, MA). K. lactis wild-type strain Y1140 (a lacI lac2) and mutant strain MS425 (a 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. [α-glucose-1-14C]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.

Abbreviations: MinLac, minimal lactose medium; YPD medium, yeast extract/peptone/dextrose medium; G418, G418 resistant.
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**Plasmids.** Plasmid pKRB1 has been described (ref. 11; Fig. 1). The other plasmids described in Fig. 1 are from Bhati (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. 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 OD500 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 [32P]oligonucleotide-labeled YRp7 cleaved with EcoRI. This plasmid has a 1.45-kilobase *S. cerevisiae* fragment, containing the TRP1 and ARS1 genes, cloned at the pBR322 EcoRI site. Autoradiograms were quantitated by densitometry and sequences hybridizing to the pBR322 portion of the probe were normalized to the two small BamHI/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 pKRB1-LAC4-1 (Fig. 1, line A) and G418-resistant (G418R) colonies were selected. Strain YNN27 was used because it transforms at high frequency, and we have used it previously for selecting G418R yeast transformants (15). Plasmid KR1B-LAC4-1 was used for reasons presented in the Discussion.

In one experiment, three G418R 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 G418R colonies or 1 per 1000 viable cells (only 25% of the viable cells were G418R). The frequency of G418R 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 G418R 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 G418R colonies even under growth conditions that select for G418R 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 replications 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 pKRB1 (Fig. 1, line F), the parent to pKRB1-LAC4-1.

**Lac+ *S. cerevisiae* Has Integrated pKRB1-LAC4-1 into a Chromosome.** To show that the Lac+ phenotype of *S. cerevisiae* is due to the presence of pKRB1-LAC4-1 and not due to the plasmid being replicating autonomously or had integrated into a host chromosome, we used Southern hybridization analysis (Fig. 2). Total DNA isolated from six independent Lac+ G418R transformants of strain YNN27 showed one band of hybridization corresponding to chromosomal DNA when the Southern blot was probed with [32P]-labeled pKRB1-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 BamHI/Bgl II-cleaved total yeast DNA. If one copy of the plasmid had integrated we would expect one of the five BamHI/Bgl II vector DNA fragments (bands, lane C) to be...
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 one gave five bands corresponding to plasmid DNA fragments plus two more intense bands (bands k and m). Thus, these five Lac− G418R transforms contained multiple tandem copies of pKRIB-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 (Fig. 1B), 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, 32P-labeled pKRIB-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 pKRIB-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 G418R 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 BamHI/Bgl II-cleaved DNA. Thirty of the 20 samples contained un integrated, 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 pi. 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 pKRIB-LAC4-1 that replicates autonomously. We predicted that the G418R 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 BamHI 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 G418R 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 G418R 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 G418R Lac− strains by plating 104–105 cells on MinLac plates. These results show that the mere presence of pKRIB-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 pKRIB-LAC4-1 Confers the Lac− Phenotype on S. cerevisiae? We assumed at this point that pKRIB-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 pKRIB-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). G418R transformants were selected and subsequently plated on MinLac plates at 104 and 105 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 pKRIB-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 lane E. We conclude from these data that a region of pKRIB-LAC4-1 (Fig. 1A) 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 pKRIB-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/pKRIB-LAC4-1 and YNN27/pKRIB-LAC4-1 and K. lactis strain MS425/pKRIB transported 85, 40, and 205 pmol of lactose per OD600 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 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, 6-O-α-D-galactosyl-D-glucose, 3-O-α-D-galactosyl-D-glucose, and 4-nitrophenyl-α-D-galactoside only weakly inhibited the permease, and thiodigalactoside showed 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.

**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 there-
fore 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 in-
creased gene dosage since Lac\(^+\) and Lac\(^-\) YNN27 transform-
ants 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\(^\circ\) phenotype is stable in
YNN27/pKR1B-LAC4-1 while the Lac\(^+\) phenotype is un-
stable. 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 se-
quences 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 selec-
tion 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 re-
search.

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