Transformation of *Kluyveromyces fragilis*

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Transformation of different yeast species is gaining importance due to their potential usefulness as hosts for cloning and expression of both native and foreign genes with the help of recombinant DNA techniques. To date, successful transformation systems have been reported for yeast species *Saccharomyces cerevisiae* (2, 8, 15), *Schizosaccharomyces pombe* (1), and *Kluyveromyces fragilis* (5, 6). Here we describe the conditions and vectors for the transformation of wild-type strains of *Kluyveromyces fragilis*, a biotechnologically important yeast species which is related to *K. lactis* but, unlike *K. lactis*, can grow over a wide range of temperatures (17). Most of the transformation systems depend on the use of auxotrophic markers for the selection of transformants. For many of the industrial yeast species, auxotrophic markers are often not available, and this poses a problem for their transformation. Transformation of *S. cerevisiae* without the use of auxotrophic markers has been achieved by using the bacterial β-lactamase gene or the kanamycin resistance (Km') determinant of Tn501 (9, 11) as markers. The kanamycin resistance gene encodes a phosphotransferase which can inactivate the aminoglycoside antibiotic G418 (11). It should, therefore, be possible to transform a wild-type yeast strain to antibiotic G418 resistance if the organism is sensitive to this antibiotic and able to express a Km' determinant. In addition to the selectable marker, a suitable replicon is required as part of the transforming vector. Previously, we have shown that 2-μm DNA of *S. cerevisiae* is a poor replicon for *K. lactis* and that ARS1 of *S. cerevisiae* transforms *K. lactis* only at a low frequency and predominantly by integration into the genome (5, 15).

High-frequency transformation of *K. lactis* was made possible by cloning autonomously replicating sequences (called KARS) from this yeast (5). Since *K. lactis* and *K. fragilis* are closely related, we considered it likely that some of the KARS sequences may serve as replicons in *K. fragilis*. KARS2 was especially promising since it allows autonomous replication in *S. cerevisiae*.

To transform a wild-type strain of *K. fragilis* to antibiotic G418 resistance, we constructed plasmid pGL2 (Fig. 1), which has the Km' gene of bacterial plasmid pACYC177 (4) and a BglII fragment containing the TRP1 gene and KARS2 subcloned from pKARS2 (5). The sensitivity of *K. fragilis* to antibiotic G418 was tested both on richYPD medium (1% yeast extract, 2% peptone, 2% glucose [pH 6.0]) and minimal YNB (yeast-nitrogen base) medium. It was found that, on the rich medium, wild-type *K. fragilis* C21 is sensitive to antibiotic G418 at a concentration of 50 μg/ml, whereas on the minimal medium it is still resistant to the antibiotic even at a concentration of 500 μg/ml. In all our experiments we have used YPD medium for the selection of transformants on antibiotic G418.

The procedure of transformation used is based on the treatment of intact yeast cells with alkali metal cations as described previously for *S. cerevisiae* (10). We wanted to test whether this method is also applicable to yeast species other than *S. cerevisiae*. The yeast cells were grown aerobically in 15 ml of YPD medium to an optical density of 3 to 6 at 590 nm. The cells were centrifuged, washed once with TE buffer (50 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA), and suspended in the same buffer to a concentration of 2 x 10^8 cells per ml. A 0.5-ml portion of this cell suspension was transferred to a test tube, and an equal volume of 0.2 M LiCl was added. After 1 h of incubation at 30°C with gentle shaking, 0.1 ml of the cell suspension was mixed with 10 μg of plasmid DNA and incubated at 30°C for 30 min. An equal volume of 70% polyethylene glycol 4000 was then added and mixed thoroughly by vortexing. After 1 h of incubation at 30°C followed by 5 min at 42°C, the cells were immediately cooled by adding 1 ml of water at room temperature, washed twice with water, and finally suspended in 100 μl of water. Ten minutes before spreading the cells, 10 ml of YPD agar (1.8%) containing 100 μg of antibiotic G418 per ml (for *K. fragilis*) was overlaid with 10 ml of YPD agar without antibiotic, leading to a final concentration of 50 μg/ml. For *K. lactis*, the starting concentration of antibiotic G418 was 150 μg/ml, and the final concentration was 75 μg/ml. Aliquots of the cell suspension were plated on solidified agar.

For the selection of Trp' transformants of *K. lactis* SD11, suitable aliquots of the final cell suspension were spread on minimal YNB containing 1% glucose. For the selection of Trp' transformants of *S. cerevisiae* YNN27, the minimal medium was supplemented with uracil (20 μg/ml). Both antibiotic G418 resistance and Trp' transformants appeared after 48 h of incubation at 30°C.

The stability of transformants was determined by growing the cells in YPD without antibiotic G418 for 10 generations and then checking their ability to grow on YPD containing antibiotic G418 (150 μg per ml) or on tryptophan-deficient minimal medium.

The results of the transformation experiments are presented in Table 1. For the selection of *K. fragilis* transformants,

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antibiotic G418 was used at a final concentration of 50 µg/ml. We obtained 20 antibiotic G418-resistant transformants per 10 µg of plasmid DNA. No spontaneously resistant colonies appeared at this concentration. Agar containing 100 µg of antibiotic G418 per ml was prepared, and, shortly before plating the transformed yeast cells, the agar layer was overlaid with medium containing no antibiotic to give a final concentration of 50 µg/ml. This allows the expression of the Km' gene before the cells come in contact with the antibiotic. No transformants were obtained when the cells were spread on the plate containing the antibiotic at a homogeneous concentration of 50 µg/ml. Probably the antibiotic inhibits protein synthesis before the Km' gene is expressed and confers resistance.

Wild-type K. lactis CBS2360 was transformed to antibiotic G418 resistance with pGL2 by the same procedure, except that the concentration of the antibiotic was 75 µg/ml. On the average, 25 transformants were obtained with 10 µg of plasmid DNA. After selection at the concentration mentioned above, both K. fragilis and K. lactis transformants were resistant to antibiotic G418 at 300 µg/ml.

The autonomous existence of the plasmid could be demonstrated in the transformed cells of both K. fragilis and K. lactis (Fig. 2). Southern blots of undigested and BamHI-digested DNA of transformants were hybridized with 32P-labeled pACYC177 DNA, the bacterial part of plasmid pGL2. The hybridizing bands in lanes 5 and 6 of Fig. 2 (K. fragilis) have electrophoretic mobilities corresponding to the supercoiled and open circular forms of the transforming plasmid in lane 2 and its linearized form after BamHI digestion in lane 1. The transforming plasmid in lane 2, isolated from Escherichia coli, contains an additional oligomeric band. After linearization with BamHI, the K. fragilis DNA showed a single band at the position of the linear plasmid DNA (lanes 7 and 8). Untransformed K. fragilis did not contain any hybridizing band (lanes 3 and 4). As a control, the DNA from untransformed and transformed K. lactis (lanes 9 to 14) is shown to have the expected band.

<table>
<thead>
<tr>
<th>Strain transformed</th>
<th>Genotype</th>
<th>Phenotype selected</th>
<th>No. of transformants/10 µg of plasmid DNA</th>
<th>Loss of antibiotic G418 resistance or Trp+ phenotype after 10 generations of nonselective growth (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K. fragilis C21</td>
<td>Wild</td>
<td>G418 resistance</td>
<td>20</td>
<td>71</td>
</tr>
<tr>
<td>K. lactis CBS2360</td>
<td>Wild</td>
<td>G418 resistance</td>
<td>25</td>
<td>78</td>
</tr>
<tr>
<td>K. lactis SD11</td>
<td>lac4 trp1</td>
<td>Trp+</td>
<td>1,500</td>
<td>65</td>
</tr>
<tr>
<td>S. cerevisiae YNN27</td>
<td>ura3 trp1 gal2</td>
<td>Trp+</td>
<td>800</td>
<td>90</td>
</tr>
</tbody>
</table>

FIG. 1. Construction of pGL2. A 3.3-kilobase BglII fragment containing the TRPI gene of S. cerevisiae and the functional KARS2 sequence was subcloned from plasmid pKARS2 (5) into the BglII site of YRp7 (details of subcloning not described). The resulting plasmid pEK27 offers the possibility of using the restriction sites Sphl, BamHI, ClaI, EcoRI, or BglII for inserting the KARS2 TRPI-containing fragment into another plasmid. To construct plasmid pGL2, pK21 DNA digested with BamHI and pEK27 DNA digested with BglII were mixed and ligated. E. coli JA300 (thr leu B6 thi thyA trpC117 hsr hsm, Str' [16]) was transformed to Km'. The Km' transformants were replica plated on minimal M9 medium containing ampicillin (100 µg/ml) and supplemented with threonine, leucine, thiamine, and thymine. DNA from Ap' Trp+ colonies were analyzed. pGL2 was isolated from one such Ap' Km' Trp+ colony.
pattern. The existence of the plasmid as free molecules in the transformed cells was confirmed by transforming E. coli to Km' or Ap' with total DNA of the yeast transformants and isolation of pGL2 as analyzed by different restriction enzymes (data not shown). As expected, the transformants are unstable under nonselective conditions. When the antibiotic G418-resistant transformants were allowed to grow in a medium without the antibiotic for 10 generations, 70 to 80% of the cells lost their resistance. Therefore, we conclude that plasmid pGL2 replicates autonomously in K. fragilis and K. lactis, although the frequency of transformation is low. Apparently, the low frequency is due to the antibiotic G418 selection. When strain SD11, a trp1 auxotroph of K. lactis (5), was transformed with pGL2 and the TRP1 gene was used as the selective marker, 1,500 Trp+ transformants per 10 μg of plasmid DNA were obtained. Twenty randomly chosen Trp+ transformants were all found to be resistant to antibiotic G418. The low frequency of transformation when selected on antibiotic G418 is therefore attributable to the selective marker used and not to the procedure of transformation or to the replicating efficiency of KARS2. In fact, KARS2 proved to be a more versatile replicon than ARSI of S. cerevisiae. When S. cerevisiae YNN27 (14) was transformed with pGL2, we obtained 800 Trp+ transformants with 10 μg of plasmid DNA. It has been reported earlier that ARSI of S. cerevisiae does not replicate in K. lactis (5).

It is clear that plasmid pGL2 is able to transform K. fragilis, K. lactis, and S. cerevisiae and to replicate autonomously in those species. The selection of transformants of wild-type strains from all three species is possible by the use of the Km' gene on pGL2. Furthermore, the results show that the method of transformation of intact yeast cells as described for S. cerevisiae by Ito et al. (10) can be successfully applied also to K. fragilis and K. lactis.

**LITERATURE CITED**


