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Genetic engineering of baker's and wine yeasts using formaldehyde hyperresistance-mediating plasmids

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Abstract

Yeast multi-copy vectors carrying the formaldehyde-resistance marker gene *SFA* have proved to be a valuable tool for research on industrially used strains of *Saccharomyces cerevisiae*. The genetics of these strains is often poorly understood, and for various reasons it is not possible to simply subject these strains to protocols of genetic engineering that have been established for laboratory strains of *S. cerevisiae*. We tested our vectors and protocols using 10 randomly picked baker's and wine yeasts all of which could be transformed by a simple protocol with vectors conferring hyperresistance to formaldehyde. The application of formaldehyde as a selecting agent also offers the advantage of its biodegradation to CO_2 during fermentation, i.e., the selecting agent will be consumed and therefore its removal during down-stream processing is not necessary. Thus, this vector provides an expression system which is simple to apply and inexpensive to use.

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- Yeast
- Transformation
- Hyperresistance to formaldehyde

Introduction

Research on the molecular biology of highly specialized industrial yeast strains is hampered by the fact that their genetics is poorly known, i.e., one can find genome organization ranging from diploid to polyploid. The construction of auxotrophic strains which are a prerequisite for transformant selection as performed in academic research is hindered by polyploidy, homothallism or even aneuploidy of industrial yeast strains. Classical genetic techniques such as strain crossing followed by tetrad analysis cannot be applied because of low sporulation, poor spore viability and homothallism resulting in the appearance of diploid cells in haploid cultures; even if four spores survive they may be infertile and unfit for further genetic analysis (1). Most of the naturally occurring yeasts are homothallic, i.e., haploid spores change their mating types regularly and after fusion with sister cells continue to grow as diploids (for a review, see Ref. 2). For these reasons, dominant selection markers are necessary for successful transformation of these strains. Some vectors based on dominant marker genes for selection of transformed cells have been established in recent years, which mediate resistance against amino acid analogs (3,4), antibiotics (5,6) or copper ions (7). In 1986, we described yeast multi-copy formaldehyde (FA) hyperresistance as a selection marker (8). This hyperresistance phenotype is achieved by overexpression of the *S. cerevisiae* gene *SFA*, coding for a glu-

tathione-dependent formaldehyde dehydrogenase (9). Consequently, this led to the development of the formaldehyde selectable yeast vector YFRp1 (10). We demonstrate here the applicability of this vector and derivatives in genetic engineering of baker's and brewer's yeasts which paves the way for the development of simple to apply and inexpensive to use expression vectors for these hosts.

Material and Methods

Strains

Ten industrial strains of *Saccharomyces cerevisiae* were obtained from Europe and North and South America. YPH98 (*MAT*a, *ade2-101*, *lys 2-801*, *leu2-Δ1*, *trp1-Δ1*, *ura3-52* (11)) was used as a standard laboratory strain of *S. cerevisiae.*

Yeast transformation protocols for YFRp plasmids

Electroporation. Laboratory yeast strains were transformed by means of a modified electroporation protocol (12). Cells were collected from an agar plate, washed with and resuspended in 1 M sorbitol at a concentration of $>10^9$ cells/ml. One µg transforming DNA and 10 µg carrier DNA dissolved in up to 10 µl H_2O were added to 50 µl of this suspension before electroporating the cells at 1.5 kV in a 2-mm cuvette (Biorad, Munich, Germany). Cells were resuspended in 1 ml YPD medium, incubated overnight at 30°C without shaking and plated onto YPD medium containing 5 mM FA.

Lithium acetate transformation. For transformation of yeast a lithium acetate protocol (13) was modified as suggested by Wehner and Brendel (10). Cultures were harvested in the exponential growth phase and concentrated to 2×10^9 cells/ml in 0.1 M lithium acetate, 0.1 M Tris and 50 mM EDTA, pH 7. Fifty µg sonicated salmon sperm DNA and 1 µg transforming DNA dissolved in up to 20 µl H_2O were added to aliquots of 50 µl of the cell suspension. After addition of 300 µl 50% PEG5000 the suspension was mixed and incubated for 1 h at 30°C. After heating the suspension to 42°C for 15 min, cells were collected (6000 *g*, 5 min) and suspended in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose) at a concentration of 2×10^6 cells/ml. Cells were incubated overnight at 30°C without shaking. Selection of transformants was carried out by plating the overnight cultures onto dishes containing 5 or 15 mM FA.

Spheroplast transformation. Yeast cells were alternatively transformed by a modified spheroplast transformation protocol (14). One-hundred-ml yeast cultures were harvested in the exponential growth phase and washed with and resuspended in 5 ml 1 M sorbitol. This suspension was incubated with 5 µl ß-mercaptoethanol and 1 mg zymolyase (US Biological, Swampscott, MA) at 30°C under gentle shaking until >90% of cells burst when incubated in distilled water. Spheroplasts were washed twice with 1 M sorbitol and then washed with and resuspended in 1 ml 1 M sorbitol, 10 mM Tris-HCl, pH 7.4, and 10 mM $CaCl_2$. Ten µl carrier DNA (50 mg/ml), 1 µg transforming DNA dissolved in 5 µl H_2O and 1.5 ml of 45% (w/v) polyethylene glycol 3350, 10 mM Tris-HCl, pH 7.4, and 10 mM $CaCl_2$ solution were added to 150 µl of this cell suspension. After a 10-min incubation at room temperature, cells were collected, resuspended in 0.5 ml 1 M sorbitol, 10 mM Tris-HCl, pH 7.4, and 10 mM $CaCl_2$ and embedded in 10 ml liquid regeneration agar (0.5% w/v ammonium sulfate, 0.17% w/v yeast nitrogen base, 2% w/v glucose, 3% w/v agar, 0.02% w/v sodium hydroxide, 2% v/v liquid YPD and 1 M sorbitol at 55°C) on YPD plates and incubated overnight at 30°C. Slices of this regeneration agar were cut and incubated overnight in liquid YPD medium containing 20 mM FA. Single transformant colonies were

obtained by plating dilutions of this overnight culture onto YPD dishes containing 15 mM FA.

Determination of formaldehyde degradation. Degradation of FA was measured by a colorimetric assay (15). Samples were incubated with equal volumes of Hantzsch reagent (2 M ammonium acetate, 50 mM acetic acid, 20 mM acetyl acetone) for 1 h at 36°C and assayed photometrically at 412 nm.

Determination of growth inhibition and plasmid loss. For determination of growth inhibition liquid cultures containing 1 x 10^6 cells/ml were incubated in the presence of different concentrations of FA in YPD with shaking at 30°C. At the beginning of the experiment and after 24 h of growth in the presence of FA, cells were counted under a microscope to obtain the total number of cells. To determine the number of viable cells, appropriate dilutions were plated onto YPD medium and incubated at 30°C for 3 days. For determination of plasmid loss, liquid cultures of transformed wine yeasts were grown in YPD medium lacking FA. After 50 generations, appropriate dilutions of the cell suspension were plated onto solid YPD. After 3 days of growth at 30°C, colonies were replicated onto dishes containing 15 mM FA in solid YPD medium for the evaluation of the plasmid-containing fraction.

Qualitative determination of ß-galactosidase activity. ß-Galactosidase (ß-Gal) activity was qualitatively determined on colonies grown on YPD plates containing 50 mg/l 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal). Cells were permeabilized by applying a drop of breaking buffer (2% v/v Triton X-100, 1% w/v sodium dodecylsulfate, 100 mM NaCl) onto the colonies.

Results

Plasmid constructions

All vector constructs are shown in Figure 1. For construction of the promoter-containing vector YFRp20 a 430-bp PCR fragment containing the upstream region of nucleotides -430 to -1 of the *ADH1* gene was inserted into the cloning site of vector YFRp1 (10) at the *Eco*RI site. To test the functionality of the *ADH1* promoter sequence the 950-bp *Pst*I/*Hin*dIII *ura3** fragment, containing the structural gene but not the promoter, was isolated and ligated into vectors YFRp1 and YFRp20, yielding vectors YFRp1U and YFRp20U, respectively. For demonstration of heterologous gene expression the 3.3-kb *Hin*dIII/*Xba*I *HNM1* promoter ß-galactosidase fragment was isolated from vector pZL4021 (16) and ligated into vector YFRp1 to yield vector YFRp1*ß-Gal*. To increase hyperresistance to FA, vector YFRp10 was constructed by inserting a 4.3-kb *Bam*HI fragment containing *ADH1* and a 3.7-kb *Hin*dII/*Pst*I fragment containing *SFA* into the multiple cloning site of vector YEp352 (17).

Introduction of DNA into various kinds of yeast and selection of transformants

All yeast strains tested exhibited an increased FA tolerance when transformed with vectors containing the *SFA* gene. As shown in Table 1 and Figure 2, FA tolerance rose by a factor of 5, from 1 mM to 5 mM for the haploid laboratory strain YPH98 and from approximately 4 to 20 mM for the industrial production strains of unidentified genetic background. For the selection of transformants, agar plates with FA concentrations of 3 mM for laboratory strains or 15 mM for wine and baker's yeasts, respectively, were shown to be optimal. Highly recommended is the use of sonicated carrier DNA in the transformation procedure, which resulted in an approximate ten-fold increase in transformation efficiency. Since transformation efficiency differed from one strain to another it might be necessary to try different protocols for optimal transformant yield.

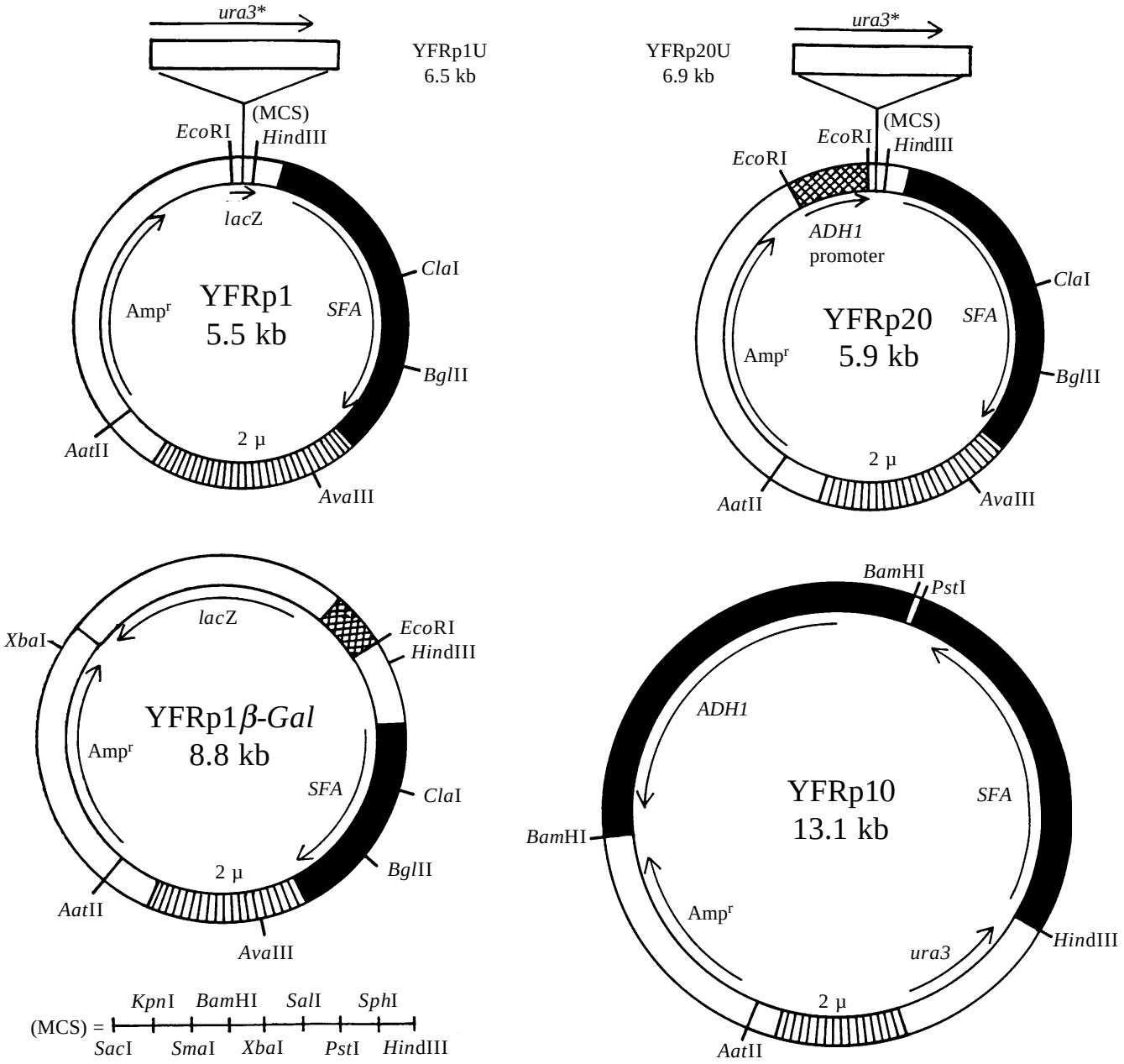

Figure 1 - Plasmids constructed for this study. Dark regions mark resistance genes, cross-hatched regions indicate *ADH1* or *HNM1* promoter sequences, respectively. The truncated *ura3** gene was inserted into the promotor-less (YFRp1) and the *ADH1* promotor-containing (YFRp20) FA-selectable vectors to yield vectors YFRp1U and YFRp20U, respectively, of which only the last one conferred uracil-prototrophy on FA-resistant transformants. A ß-galactosidase/*HNM1* promotor fusion construct was ligated into vector YFRp1 yielding plasmid YFRp1*ß-Gal* which conferred ß-galactosidase activity on transformants. YFRp10 contains two FA-resistance factors, *SFA* and *ADH1*, ligated into vector YEp352.

Characterization of transformants

Heterologous gene expression of transformed cells was demonstrated with vectors YFRp1U, YFRp20U and YFRp1*ß-Gal*. The cells transformed with vector YFRp1*ß-Gal* exhibited a pronounced ß-galactosidase activity that led to a blue color when colonies grown on YPD plates containing X-Gal were incubated with breaking buffer, whereas cells transformed with the control plasmid YFRp1 stayed white. The functionality of the *ADH1* promoter was proven by transforming the uracil-auxotrophic laboratory strain YPH98 with vector YFRp20U. Transformants were first selected regarding FA resistance and then transferred onto synthetic media lacking uracil to test for expression of the *ura3** reading frame. Cells transformed with vector YFRp20U were able to grow whereas cells transformed with vector YFRp1U (lacking the *ADH1* promoter) were not, indicating that *ura3** was expressed with the help of the *ADH1* promoter. Both transformants were controlled by isolating the respective plasmid from the transformed cells and, in case of YFRp20U, by a plasmid loss experiment in which cells that lost the plasmid-encoded FA hyperresistance also exhibited uracil-auxotrophy again. Plasmid loss was determined using YFRp1-transformed wine yeast strains and occurred after 50 generations of growth in the absence of FA in 60-75% of the cell population.

Table 1 - Suitable transformation protocols and tolerated formaldehyde concentrations (mM) of transformed yeast strains.

E = Electroporation, L = lithium acetate protocol, S = spheroplast method.

	Suitable transformation protocol	YEp24	YFRp1	YFRp20	YFRp10
YPH98	E, L, S	1	5	5	6
9 wine yeast strains	L, S	4	20	20	25
1 baker's yeast strain	S	4	20	20	25

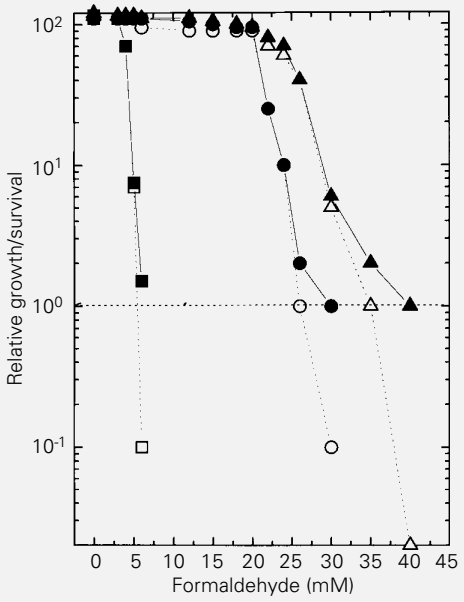

Figure 2 - Growth inhibition and survival of one wine yeast strain transformed with YFRp1 (circles), YFRp10 (triangles), and the non-transformed control (squares) after exposure to FA. Values for relative growth and survival are given after 24 h of growth in FA-containing YPD medium in relation to the respective start values. Solid lines/filled symbols indicate total number of cells and dashed lines/open symbols the fraction of viable cells.

FA degradation

The selective agent was almost completely metabolized to CO_2 by the yeast culture: after only 24 h the FA content decreased by 95% from 5 mM to 0.25 mM in liquid cultures of the transformed haploid laboratory strain (data not shown).

Discussion

The alteration of physiological properties of industrially employed strains of *Saccharomyces cerevisiae* by means of genetic engineering is an expanding field of research, mainly dealing with problems of ameliorating the products of fermentation processes or with producing complex substances inside transformed cells. These industrially applied yeast strains are wild types for practically all genes encoding proteins of metabolic pathways and have poorly defined genotypes since they have been selected over the years for top performance in specialized tasks.

Lacking auxotrophy markers, they require dominant selection markers for genetic transformation (for a review, see Ref. 1). While stable integration of new genes into the genome of the production strains is mainly used to alter their fermentation characteristics (18), transformation of cells with multicopy vectors is most suitable to achieve overproduction of desired substances (e.g., 19). For this aim the genes of interest are transferred into target cells as part of selectable multi-copy vectors, often fused to promoter sequences which either allow conditional or highly constitutive expression of the cloned gene.

Some suitable marker genes for selection of transformed cells have been established in recent years, which mediate resistance against amino acid analogs (3,4), antibiotics (5,6) or copper ions (7). In the present study we show the applicability of another selection system that seems to be advantageous for large-scale fermentations, because unlike most of the above-mentioned selection procedures, it requires an inexpensive chemical that is subject to degradation during the fermentation process of FA-hyperresistant yeast cultures. Selection based on FA hyperresistance allows growth of yeast transformants in the usual industrial media to which they are optimally adapted, with the selective agent being metabolized mainly to CO_2 and to traces of methanol (10,20). Thus, the use of FA reduces and sometimes practically eliminates the problem of removal (detoxification) of the employed selective chemical FA (poison, weak mutagen; 21). This is a result of the plasmid persistence in transformed cells after gradual lowering of the selective pressure which guarantees further removal of the remaining formaldehyde from the medium even at low non-selective concentrations. Thus, the industrial application of FA hyperresistance-conferring plasmids would require two stages: 1) transformant cell propagation in the presence of 20 mM FA (0.06% w/v) and, a few generations before reaching the desired cell mass, 2) growth without further addition of FA, thus permitting elimination of FA by oxidative metabolism followed by the proper production process in the absence of FA. FA-containing genotoxic fumes should be kept in closed fermentation systems and safe down-stream processing should be used to prevent health hazards due to the remaining traces of FA.

The *SFA* gene used in this study is derived from *S. cerevisiae* itself, so that there is no problem for homologous expression. When the desired production gene has been cloned into our vectors, the *E. coli* pBR322-derived ampicillin resistance marker should be eliminated so that antibiotic resistant markers would not be spread in the product or in the waste; also, elimination of pBR322 sequences would reduce vector size resulting in larger copy numbers in the transformants. In 1993, we also showed that the genotoxicity of the selective chemical FA is drastically reduced in FA-hyperresistant yeast cells so that stability of plasmid constructions is insured (21). Although the efficiency of the lithium acetate transformation procedure is sufficient to obtain transformants of most yeast strains used in this study, one strain could only be transformed by applying the more time-consuming spheroplast protocol. In any case, care must be taken when selecting for transformants because some yeast strains exhibit a much slower decline of viability of non-transformed cells than that shown in Figure 2 (data not shown) with survival and cell number curves indicating a delayed growth of non-transformed cells under selective conditions, but a 100% viability of these organisms over a broad concentration range. Also, the FA-detoxifying activity of transformed cells might give rise to a strong background growth of non-transformed cells when incubated too long. Therefore, we recommend plasmid recovery and analysis of selected clones before designating them as transformants which, once determined, remain stable for years in stock cul-

tures at -70ºC.

We proved the applicability of our formaldehyde selectable vectors in the bioengineering of wild-type yeasts by showing expression and functionality of the encoded proteins. Transforming cells with the YFRp1 vector containing *SFA* confers an increased FA tolerance by a factor of five, but transforming with the vector YFRp10 yields a further increase of this tolerance because of the FA-detoxifying activity of *ADH1* (20). However, the additional expression of *ADH1* yields a further increase in FA resistance of only 20%, so that for most purposes the expression of *SFA* alone would suffice. Vector YFRp20 was constructed for heterologous gene expression, containing a strong promoter (-430 to -1 of *ADH1*, according to Ref. 22), without the *ADH1* gene's start codon, followed by a multiple cloning site. The vector allows insertion of selected genes and overproduction of desired proteins in industrial yeast cultures grown in inexpensive undefined media without constructing in-frame fusions of the respective gene and can be turned easily into an expression vector by inserting appropriate termination sequences into the cloning site or replacing the multiple cloning site by a proven expression cassette (23,24).

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