Curr Genet (1989) 15:91–98

Current Genetics

Heterologous gene expression of the glyphosate resistance marker and its application in yeast transformation

G. Kunze[1], R. Bode[2], H. Rintala[2], and J. Hofemeister[1]

[1] Zentralinstitut für Genetik und Kulturpflanzenforschung der Akademie der Wissenschaften der DDR, DDR-4325 Gatersleben, German Democratic Republic

[2] Sektion Biologie der Ernst-Moritz-Arndt-Universität Greifswald, DDR-2200 Greifswald, German Democratic Republic

Summary. The *E. coli aroA* gene was inserted between yeast promoter and terminator sequences in different shuttle expression plasmids and found to confer enhanced EPSP synthase activity as well as resistance to glyphosate toxicity. Subsequently, a transformation system using these newly constructed vectors in yeast was characterized. The efficiency of the glyphosate resistance marker for transformation and selection with plasmid pHR6/20-1 in *S. cerevisiae* laboratory strain SHY2 was found to be relatively high when compared with selection for *LEU2* prototrophy. The fate of the recombinant plasmid pHR6/20-1 in the transformants, the preservation of the *aroA E. coli* DNA fragment in yeast, mitotic stability, EPSP synthase activity, and growth on glyphosate-containing medium have been investigated. As this plasmid also allows direct selection for glyphosate resistant transformants on rich media, the glyphosate resistance marker was used for transforming both *S. cerevisiae* laboratory strain SHY2 and brewer's yeast strains *S. cerevisiae var. "uvarum"* BHS5 and BHS2. In all cases, the vector pHR6/20-1 was maintained as an autonomously replicating plasmid. The resistance marker is, therefore, suitable for transforming genetically unlabeled *S. cerevisiae* laboratory, wild, and industrial yeast strains.

Key words: *Saccharomyces cerevisiae* – Vector – Glyphosate resistance – Transformation

Introduction

When plasmids used for selecting transformants and cloning genes they most contain a dominant marker. Prototrophy markers have often been used when transforing yeast i.e., the *LEU2* (Hinnen et al. 1978), *URA3* (Struhl et al. 1979), and *TRP1* (Hitzeman et al. 1980) genes of *Saccharomyces cerevisiae*. As transformants of wild-type strains, including industrial yeasts, usually do not contain auxotrophic markers, other dominant and selective markers have been tested; these include resistance to G418 (Jimenez and Davis 1980), hygromyicin B (Gritz and Davis 1983; Kaster et el. 1984), copper (Fogel and Welch 1982), or methotrexate (Zhu et al. 1985). The low efficiency at which these systens are selected, as compared to auxotrophic markers, still creates some problems with respect to their application in yeast transformation.

Recently, the construction of a new type of resistance plasmids has resulted in high transformation efficiencies in various strains of *S. cerevisiae*. These plasmids contain genes coding for phleomycin or chloramphenicol resistance (Gatignol et al. 1987; Hadfield et al. 1987). In this paper, we describe the construction of plasmid vectors that provide resistance against glyphosate and their use in yeast transformation. The herbicide glyphosate (*N*-(phosphonomethyl-) glycine) has been found not only to be a potent inhibitor of plant growth but also a growth inhibitor of bacteria and yeast cells (Jaworski 1972; Bode et al. 1985; Bode et al. 1986). This glyphosate inhibits EPSP synthase (E.C.2.5.1.19), an enzyme which catalyzes the conversion of shikimate-3-phosphate into 5-enol-shikimate-3-phosphate. The gene coding for EPSP synthase has been cloned from *S. cerevisia* (as part of the pentafunctional *arom* polypeptide) and *E. coli* (*aroA*) (Larimer et al. 1983; Duncan et al. 1984; Duncan and Coggins 1984). In order to work in a heterologous background we have inserted the *aroA* gene of *E. coli*

Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate

Offprint requests to: G. Kunze

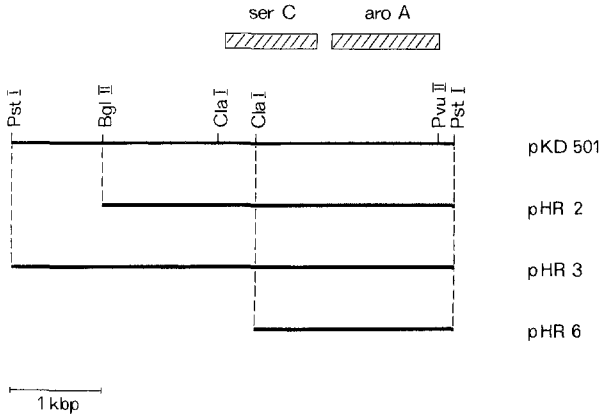

Fig. 1. Sub-cloning strategy for the *aroA* DNA from *E. coli*

into various *S. cerevisiae* plasmids and achieved its expression by yeast *ADH1* and *CYC1* promoter and terminator signals. The expression of the *aroA* gene has been monitored by measuring EPSP synthase in laboratory strains as well as in industrial strains of *S. cerevisiae*. Resistance in the presence of glyphosate has been used as suitable marker for the direct selection of yeast transformants on glyphosate media.

Material and methods

Strains and plasmids. Yeast strains used in this study were *S. cerevisiae* SHY2 (*ura3 trp1 leu2 his3 can*) (Botstein et al. 1979) and *S. cerevisiae* var. "uvarum" BHS1, BHS2, BHS4 and BHS5 (obtained from the VEB Wissenschaftlich-Technisch-Ökonomischen Zentrum der Brau- und Malzindustrie der DDR). Strains DH5 (*recA1 endA1 gyrA96 thi⁻ hsdR17 supE44*) and AB2829 (aroA⁻) (Duncan et al. 1984) were used for transforming *E. coli* and for preparing plasmid DNA. Plasmid pKD501 which carries a 4.6 kbp *E. coli* DNA fragment that includes the *aroA* gene, was used for recloning the *aroA* gene (Duncan et al. 1984). The plasmids pERIII-8PL4, pEX2, and pAAH5 have been described by Boros et al. (1986), Gritz and Davis (1983), and Ammerer (1983), respectively.

DNA preparation. Plasmid DNA from *E. coli* was isolated as described by Birnboim and Doly (1979). Total genomic or plasmid DNA was isolated from transformed yeast cells grown in minimal medium (SD) as described by Case (1983) and Kunze et al. (1984).

E. coli and yeast transformations. E. coli cells were transformed using the method of Hanahan (1983). The procedures for yeast transformation were those of Ito et al. (1983).

Enzyme preparation. Cells that had reached the stationary phase of growth were washed with 50 m*M* HEPES buffer (pH 7.5), resuspended in 50 m*M* HEPES buffer containing 1 m*M* phenylmethyl sulfonylfluoride, and disrupted by being passed twice through an X-pressure cell. A crude extract was obtained after the cell debris was removed by centrifugation at 20,000 *g* for 20 min at 4°C.

Enzyme assay. The activity of EPSP synthase (E.C. 2.5.1.19) was determined according to the method of Gollub et al. (1967). The assay mixture consisted of (in a total volume of 0.1 ml): 0.3 m*M* phosphoenolpyruvate, 0.1 m*M* shikimate-3-phosphate, 5 m*M* KF, 1 m*M* phenylmethyl sulfonylfluoride, 50 m*M* HEPES buffer (pH 7.5).

Gel filtration. Crude extracts were applied to a Sephadex G-200 column (2 × 50 cm) equilibrated with 50 m*M* HEPES buffer (pH 7.5) and containing 1 m*M* phenylmethyl sulfonylfluoride. The enzyme was eluted with the same buffer (flow rate 30 ml/h, 1.5 ml fractions).

Protein determination. Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard.

Chemicals. Shikimate-3-phosphate was prepared as described by Knowles and Sprinson (1970).

Results

Plasmid constructions

The *E. coli aroA* gene has been identified on a 4.6 kbp PstI fragment derived from plasmid pKD501. This DNA fragment is in actual fact much larger than what is required to encode the EPSP synthase, and therefore the presence of the *serC* gene also on this PstI fragment in front of the *aroA* gene (Duncan and Coggins 1986) is not surprising. In order to reclone the *aroA* gene onto different shuttle expression plasmids, various subfragments were chosen: PstI, BglII/PstI, or ClaI/PstI DNA fragments of *E. coli* were ligated into the *E. coli* plasmid pERIII-8PL4 (flanked by BamHI or HindIII sites) (Fig. 1).

Each fragment was subsequently recloned from pERIII-8PL4 into the unique BamHI or HindIII site of the yeast expression vectors pEX2 or pAAH5; this resulted in fusion with the promoter and terminator sequences of either the *CYC1* (pHR2) or *ADH1* (pHR3, pHR6/20-1) gene. Several plasmids were found to carry the *E. coli* DNA fragment in the correct orientation and were thus expected to express the *aroA* gene under control of either the *CYC1* (pHR2) or *ADH1* (pHR3; pHR6/20-1) promoter in yeast. Those plasmids were subsequently used to transform strain *S. cerevisiae* SHY2 and tested for *E. coli* EPSP synthase expression in yeast (Figs. 2, 3, 4).

Level of E. coli aroA gene expression in yeast

The transformation frequency in *S. cerevisiae* SHY2 differed significantly for plasmids pHR2, pHR3, pHR6/20-1 and pHR6/20-2 (Table 1). The lowest transformability was exhibited by plasmid pHR2. This was attributed either to the structural peculiarities of the various plasmids or to the effect of *aroA* gene expression.

EPSP synthase activity could be detected in protein extracts of the yeast transformants. In these transfor-

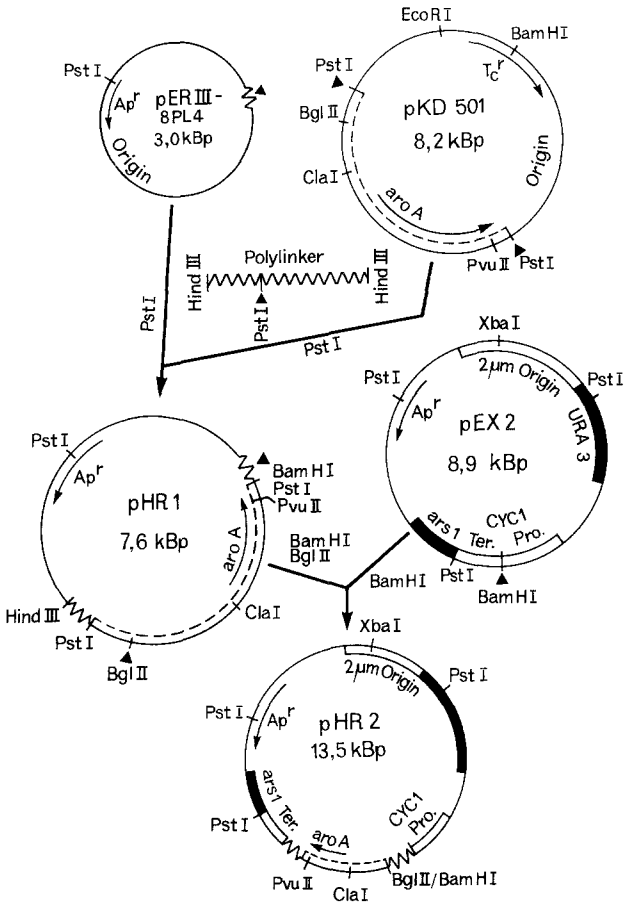

Fig. 2. Construction of the hybrid plasmid pHR2 carrying the *aroA* gene from *E. coli*. *Thick line* yeast DNA's; *thin line E. coli* plasmid DNA

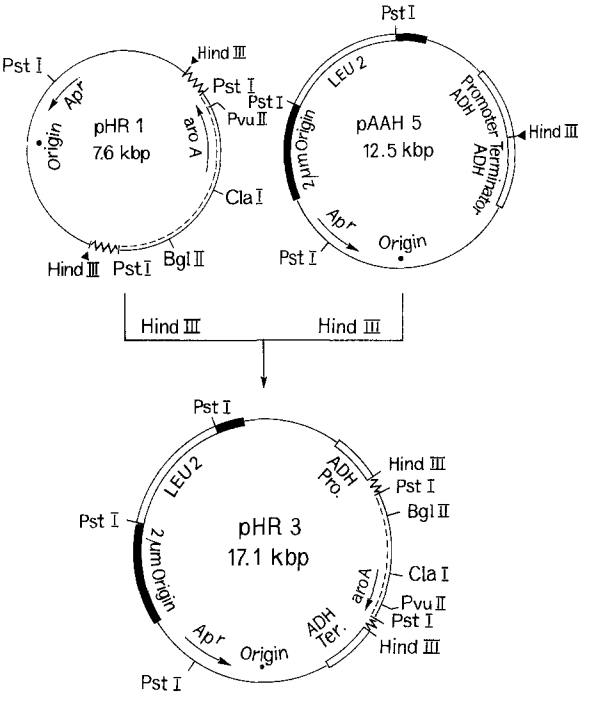

Fig. 3. Construction of the pHR3 expression plasmid. *Thick line* yeast DNA's; *thin line E. coli* plasmid DNA

mants, the assay of *E. coli* EPSP synthase was influenced by the presence of endogenous (yeast) EPSP synthase in *S. cerevisiae* SHY2. When the yeast cultures were grown in minimal medium without glyphosate the transformante and the control strain *S. cerevisiae* SHY2 displayed identical EPSP synthase activities. When the cells were cultivated in minimal medium supplemented with 2 mg/ml glyphosate, however, higher enzyme activities were measured in the transformed cells. This stimulation by glyphosate resultet in a 2.6-fold increase in EPSP synthase activity in pHR6/20-1 transformants. In contrast, our re-transformation experiments suggested that the plasmid copy number in the transformants is independent of the presence of glyphosate. *S. cerevisiae* SHY2 transformed with plasmid pHR6/20-1 showed a 3.2-fold increase in EPSP synthase relativs to *S. cerevisiae* SHY2, while yeast transformants harboring a similar plasmid (pHR6/20-2) only with the *aroA* gene in the opposite orientation between yeast promoter and terminator showed no increase in EPSP synthase activity (Table 2).

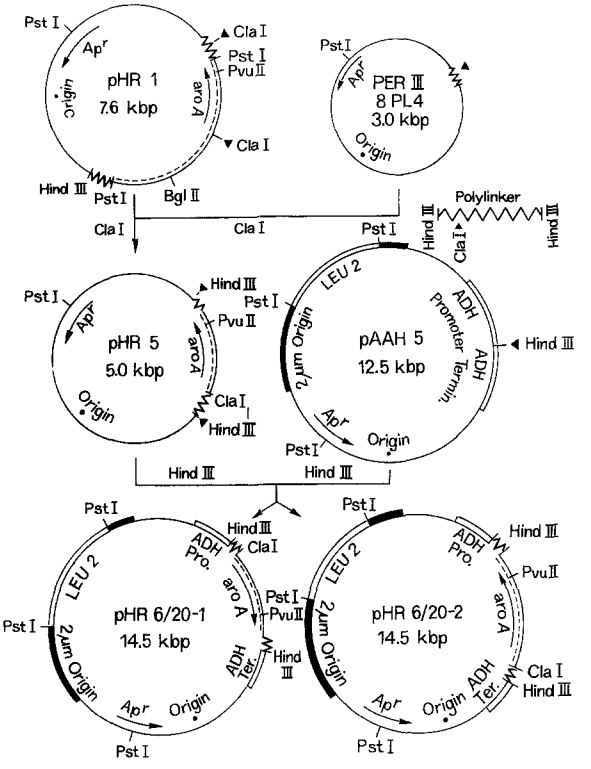

Fig. 4. Construction of the hybrid plasmids pHR6/20-1 and pHR6/20-2. *Thick line* yeast DNA's; *thin line E. coli* plasmid DNA

Table 1. Transformation frequencies of plasmids pHR2, pHR3, pHR6/20-1 and pHR6/20-2

Strain	Transforming plasmid	Transformants per μg DNA	Transformation frequency
S. cerevisiae SHY2	pHR2	22	2.2×10^{-6}
S. cerevisiae SHY2	pHR3	1,844	1.8×10^{-4}
S. cerevisiae SHY2	pHR6/20-1	896	9.0×10^{-5}
S. cerevisiae SHY2	pHR6/20-2	2,188	2.2×10^{-4}

Table 2. Specific activity (pkat/mg protein) of EPSP-synthase in *S. cerevisiae* mutants and transformants

Strain[a]	Glyphosate concentration (mg/ml)	Enzyme activity
S. cerevisiae SHY2	–	500
S. cerevisiae SHY2/pHR2	–	520
S. cerevisiae SHY2/pHR2	2.0	880
S. cerevisiae SHY2/pHR3	–	600
S. cerevisiae SHY2/pHR6/20-1	–	620
S. cerevisiae SHY2/pHR6/20-1	2.0	1,600
S. cerevisiae SHY2/pHR6/20-2	–	520
S. cerevisiae SHY2/pHR6/20-2	2.0	530

[a] Yeasts were grown in SD medium with the required supplements

In addition, on a Sephadex G-200 column it was possible to isolate both the yeast and *E. coli* EPSP synthase by their differences in molecular weights – about 300,000 and 50,000, respectively (Knowles and Springs 1970; Duncan et al. 1984) (Figs. 5, 6). Only yeast EPSP synthase could be detected in *S. cerevisiae* SHY2 transformants harboring pHR2 or pHR6/20-2 after cultivation of the transformed cells, in minimal medium without glyphosate. Only minor activity of *E. coli* EPSP synthase was measured in *S. cerevisiae* SHY2/pHR6/20-1 under such cultivation conditions. In the presence of 2 mg/ml glyphosate, *E. coli* EPSP synthase activity was found to be significantly increased in *S. cerevisiae* SHY2/pHR2 and SHY2/pHR6/20-1, but it was still absent in *S. cerecisiae* SHY2 or SHY2/pHR6/20-2.

Sensitivity of S. cerevisiae transformants to glyphosate

The minimum concentration at which glyphosate inhibited strain *S. cerevisiae* SHY2 (MIC) was 3 mg/ml, as determined in minimal medium at pH 4.0. Transformants *S. cerevisiae* SHY2/pHR2 grew in the presence of 6 mg/ml and higher concentrations of

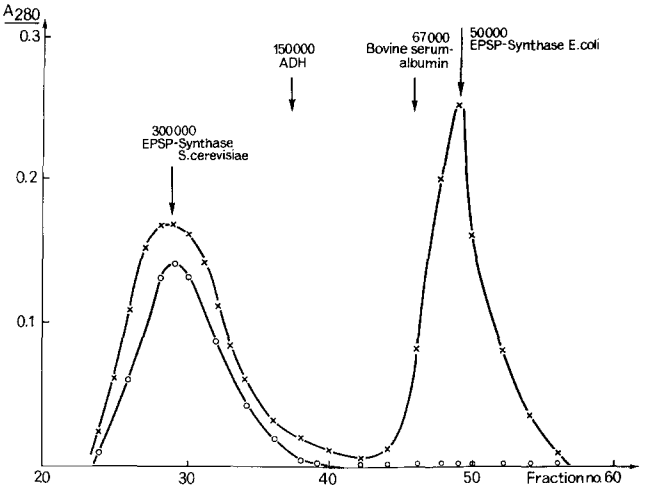

Fig. 5. Separation of the EPSP-synthases produced by *S. cerevisiae* SHY2 and *S. cerevisiae* SHY2/pHR2 on a Sephadex G-200 column. The yeast cells were grown and harvested from SD medium without and with (2.0 mg/ml) glyphosate. O—O *S. cerevisiae* SHY2/pHR2, without glyphosate; ×—× *S. cerevisiae* SHY2/pHR2, 2.0 mg/ml glyphosate

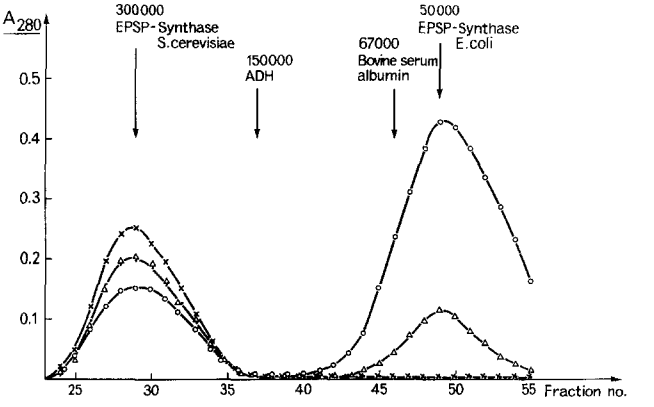

Fig. 6. Separation of the EPSP-synthases from *S. cerevisiae* SHY2/pHR6/20-1 and *S. cerevisiae* SHY2/pHR6/20-2 by Sephadex G-200 gel filtration. The yeast cells were grown in and harvested from SD medium without and with (2.0 mg/ml) glyphosate. ●—● *S. cerevisiae* SHY2/pHR6/20-2, without glyphosate; ×—× *S. cerevisiae* SHY2/pHR6/20-2, 2 mg/ml glyphosate; △—△ *S. cerevisiae* SHY2/pHR6/20-1, without glyphosate; O—O *S. cerevisiae* SHY2/pHR6/20-1, 2 mg/ml glyphosate

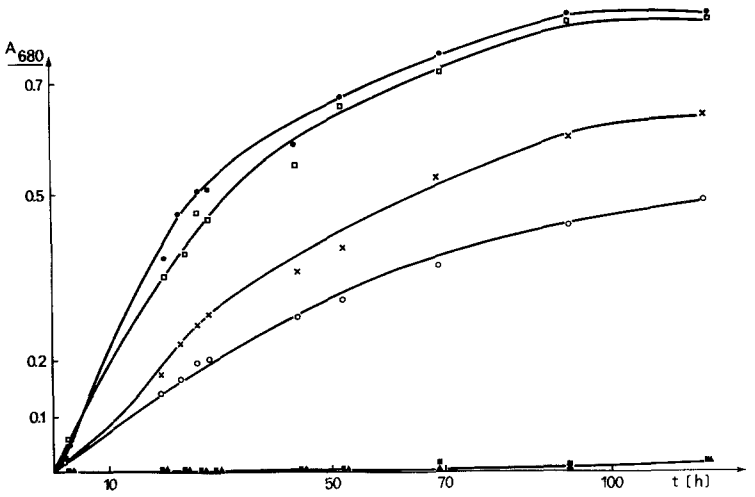

Fig. 7. Growth of *S. cerevisiae* strains in SD medium supplemented with 4.0 and 6.0 mg/ml or without glyphosate, either harboring or not harboring plasmid constructions. ●—● *S. cerevisiae* SHY2 without glyphosate; ■—■ *S. cerevisiae* SHY2 4 mg/ml glyphosate; ▲—▲ *S. cerevisiae* SHY2 6 mg/ml glyphosate; □—□ *S. cerevisiae* SHY2/pHR2 without glyphosate; ×—× *S. cerevisiae* SHY2/pHR2 4 mg/ml glyphosate; ○—○ *S. cerevisiae* SHY2/pHR2 6 mg/ml glyphosate

Table 3. Number of *S. cerevisiae* SHY2 transformants per µg DNA after selection for *LEU*$^+$ or for glyphosate resistance

Plasmid	Glyphosate concentration (mg/ml)			
	0[a]	2[b]	4[b]	6[b]
pHR3	1,844	12	4	88
pHR6/20-1	896	980	36	4
pHR6/20-2	2,188	0	96	52

[a] The LEU$^+$ transformants were selected on SD medium with the required supplements (uracil, tryptophan, histidin)
[b] The transformants were selected on rich medium with the required supplements (uracil, tryptophan, histidin, leucin)

Table 4. Number of *S. cerevisiae* SHY2 transformants per µg DNA after preselection in rich medium supplemented with 0.5 mg/ml glyphosate for 15 h and plating for selection of *LEU*$^+$ and glyphosate resistant transformants

Plasmid	Glyphosate concentration (mg/ml)			
	0[a]	2[b]	4[b]	6[b]
pHR3	1,648	0	0	0
pHR6/20-1	6,244	2,332	2,184	0
pHR6/20-2	2,552	0	12	0

[a] The *LEU*$^+$ transformants were selected on SD medium with the required supplements (uracil, tryptophan, histidin)
[b] The transformants were selected on rich medium containing glyphosate and the required supplements (uracil, tryptophan, histidin, leucin)

glyphosate (Fig. 7). On minimal solid medium (pH 6.8), this MIC was about 2.0 mg/ml. Here it was possible to cultivate the yeast transformants when the medium contained 6 or more mg/ml glyphosate.

Direct selection of transformants for glyphosate resistance

Plasmid pHR6/20-1 which conferred the highest EPSP synthase level in yeast cells, was used to optimize the conditions necessary for directly selecting glyphosate resistant transformants on minimal medium. Plasmids pHR3 and pHR6/20-2 were used as controls. When plasmid pHR6/20-1 was plated on medium containing 2 mg/ml glyphosate, it yielded approximately the same frequency of transformants as was obtained after selection for *LEU*$^+$ transformants. Transformation experiments using plasmids pHR6/20-2 and pHR3, respectively, did not result in glyphosate resistant colonies (Table 3). However, in the former, selection for glyphosate resistant transformants was superimposed by colonies showing a basic higher resistance against the herbicide. These colonies contained no plasmid, as demonstrated by re-transformation in *E. coli* and the degree of their mitotic stability. By modifying the transformation procedure, these colonies with background could be eliminated: the transformed yeast cells were pre-incubated in yeast rich medium containing 0.5 mg/ml glyphosate at 30 °C for 15 h before being plated on the glyphosate agar for selection of the transformants. When plasmid pHR6/20-1 was used, enhanced transformation frequencies were obtained even when selection occurred on selective media containing either 2.0 or 4.0 mg glyphosate per ml. Under these conditions nearly all of the colonies maintained the plasmid (Table 4). The increased transformation frequencies were probably due to cell multiplication during this incubation period. Thus using plasmid pHR6/20-1, we were able to establish a direct

Table 5. Number of transformants per μg DNA in different brewery strains of *S. cerevisiae var. uvarum*[a]

Strain	Plasmid	Glyphosate concentration (mg/ml)			
		0.5	1	2	3
BHS1	pHR6/20-1	0	1	0	0
BHS1	pHR6/20-2	0	0	0	0
BHS2	pHR6/20-1	112	88	0	0
BHS2	pHR6/20-2	0	0	0	0
BHS4	pHR6/20-1	3	0	1	0
BHS4	pHR6/20-2	0	0	0	0
BHS5	pHR6/20-1	207	171	5	0
BHS5	pHR6/20-2	8	2	0	2

[a] The transformants were selected on rich medium containing glyphosate

Table 6. Genetic stability (%) of transformants

Transformant	Selektive medium	Non-selective medium
S. cerevisiae SHY2/pHR2[a]	81,6	52,5
S. cerevisiae SHY2/pHR3[a]	80,5	50,2
S. cerevisiae SHY2/pHR6/20-1[a]	72,4	43,7
S. cerevisiae SHY2/pHR6/20-2[a]	81,3	36,2
S. cerevisiae SHY2/pHR6/20-1[b]	70,2	37,6
S. cerevisiae var. "uvarum" BHS2/pHR6/20-1[b]	76,1	34,2
S. cerevisiae var. "uvarum" BHS5/pHR6/20-1[b]	68,5	40,3

[a] Transformants were first grown in minimal medium (without leucine) under selective conditions or in rich medium under non-selective conditions and aliquots were plated after 10 generations on selective (without leucine) or non-selective (with leucine) agar plates
[b] Transformants were cultivated in minimal medium containing additional leucine and 2 mg/ml glyphosate under selective conditions or in minimal medium with leucine but without glyphosate under non-selective conditions and aliquots were plated after 10 generations on selective (with leucine and 2 mg/ml glyphosate) or non selective (with leucine but without glyphosate) agar plates

selection system for yeast transformation using glyphosate resistance as a marker.

Transformation of industrial yeast strains

The plasmids pHR6/20-1 and pHR6/20-2 were used for transforming industrial *S. cerevisiae var. "uvarum"* strains, which are commonly used in the brewing industry. Since these strains have no auxotrophic markers, transformation was measured by the use of the glyphosate resistance marker. The brewer's yeast strains *S. cerevisiae var. "uvarum"* BHS1, BHS2, BHS4, and BHS5 were initially tested for basic glyphosate sensitivity in minimal solid medium (pH 6.8) and found to be sensitive to 0.4 mg/ml (BHS5) or 0.3 mg/ml (BHS1, BHS2, BHS4). When plasmid pHR6/20-1 was used, transformation frequencies were significantly lower using brewery strains BHS2 and BHS5 than with laboratory strain *S. cerevisiae* SHY2 (Table 5). Up to now, we have been unable to transform the other under the transformation conditions used in this study.

Table 7. Retransformation of *E. coli* with DNA isolated from yeast transformants supposed to harbor plasmids pHR2, pHR3, pHR6/20-1 and pHR6/20-2

DNA source[a]	Transformants per μg DNA	
	Ap^r	Ap^r, $aroA^+$
S. cerevisiae SHY2[b]	0	0
S. cerevisiae SHY2/pHR2[b]	36	36
S. cerevisiae SHY2pHR3[b]	48	48
S. cerevisiae SHY2/pHR6/20-1[b]	44	44
S. cerevisiae SHY2/pHR6/20-2[b]	76	76
S. cerevisiae SHY2/pHR6/20-1[c]	56	56
S. cerevisiae var. "uvarum" BHS5[b]	0	0
S. cerevisiae var. "uvarum" BHS5/pHR6/20-1[c]	27	27
S. cerevisiae var. "uvarum" BHS2[b]	0	0
S. cerevisiae var. "uvarum" BHS2/pHR6/20-1[c]	12	12

[a] Yeast DNA (5 μg) was isolated by the standard method described by Case (1983) and was transformed in *E. coli* AB2829 (Hanahan 1983)
[b] Yeast cells were cultivated in minimal medium enriched by required supplements but without glyphosate
[c] Yeasts were grown in minimal medium with 2 mg/ml glyphosate and the required supplements

Characterization of the yeast transformants

The frequent segregational loss of leucine prototrophy (pHR2, pHR3, pHR6/20-1, pHR6/20-2) or glyphosate resistance pHR6/20-1 from all transformants but especially those grown under non-selective conditions, suggested that all these transformants maintain autonomously replicating plasmids (Table 6). Corroborative evidence for autonomously replicating plasmids was obtained by the re-transformation of *E. coli* AB2829 with DNA of these transformants using the ampicillin resistance and $aroA^+$ selection marker of the vector plasmid. *E. coli* transformants were recovered in every case. All the selected ampicillin resistant colonies were also $aroA^+$ (Table 7). The plasmid DNA from these transformants was isolated and analyzed by agarose gel electrophoresis. All plasmids recovered were found

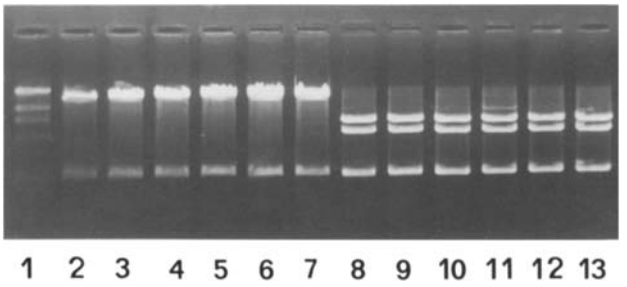

Fig. 8. Electrophoresis of HindIII (*lanes 2–7*) and EcoRI (*lanes 8–13*) fragments of pHR6/20-1 DNA in an 0.8% agarose gel. pHR6/20-1 (*lanes 2* and *8*) was obtained from *E. coli* AB2829; plasmid DNA recloned in *E. coli* AB2829 was obtained from *S. cerevisiae* SHY2/pHR6/20-1 (*lanes 3* and *9*), *S. cerevisiae var. "uvarum"* BHS5/pHR6/20-1 (*lanes 4, 5, 10* and *11*) and *S. cerevisiae var. "uvarum"* BHS2/pHR6/20-1 (*lanes 6, 7, 12* and *13*). HindIII digested lambda DNA was used as standard (*lane 1*)

to have the predicted restriction patterns without DNA rearrangements or deletions (Fig. 8).

Discussion

Various plasmid constructions (pHR2, pHR3, pHR6/20-1) carrying the *E. coli aroA* gene in fusion with either yeast *CYC1* or *ADH1* promoter and terminator sequences were found to express the *E. coli aroA* gene in *S. cerevisiae*. The levels at which this gene was expressed were strictly dependent on *E. coli* DNA sequences separating the yeast promoter and the translation start of the *aroA* gene. The activity of EPSP synthase in transformants was low in experiments using pHR3 and higher when pHR6/20-1 was used. The latter plasmid contains the shortest for all the *E. coli* DNA sequences intervening between the yeast promotor and the translation start of the *aroA* gene.

The *E. coli* EPSP synthase in yeast is adaptive, i.e., enzyme activity in yeast transformants appears immediately following cultivation of the transformants in minimal medium containing glyphosate. The adaptivity of the EPSP synthase is still a matter of speculation: glyphosate may either induce the expression of the *aroA* gene in yeast or EPSP synthase could be prevented from degradation. The latter pathway has been described for plant cells (Hollaender-Czytko et al. 1988). The glyphosate resistance coded and expressed by plasmid pHR6/20-1 was found to be at a high enough level as to allow the direct selection of transformants on solid glyphosate media, while transformants carrying other plasmid constructions expressed low EPSP synthase activity and did not show resistance. When plasmid pHR6/20-1 was used transformant selection on glyphosate medium was improved when the transformed cells were pre-incubated for a 15-h period in the presence of a low concentration of glyphosate. Under such conditions it was possible to transform *S. cerevisiae var "uvarum"* industrial (brewing) yeast strains BHS2 and BHS5 and to select for glyphosate resistance. The efficiency of the transformation (and selection) system using plasmid pHR6/20-1 was established for various yeast strains and subsequently compared with selection efficiency using the *LEU2* prototrophy marker. Our results correspond to those obtained by Gatignol et al. (1987) and Hadfield et al. (1987) who used phleomycin and chloramphenicol resistance plasmids. In plasmid pHR6/20-1 transformants of *S. cerevisiae* SHY2 and *S. cerevisiae var. "uvarum"* BHS2 and BHS5, either selected for by amino acid prototrophy (LEU^+) or glyphosate resistance, the plasmids always existed autonomously. This was evident from their mitotic stability pattern and retransformation into *E. coli*.

We suggest that our plasmid vectors are useful for the co-transformation of DNA coding for an industrial important trait and, therefore, can be used for the genetic manipulation of industrial yeasts. Futher experiments are in progress which we hope will demonstrate the glyphosate resistance marker to be a useful tool in the genetic engineering of genetically unlabeled laboratory, wild, and industrial yeast strains.

Acknowledgements. We are grateful to Professors J. R. Coggins (Glasgow), P. Venetianer (Szeged) and C. P. Hollenberg (Düsseldorf) for providing *E. coli* strains and plasmids. We wish to thank Professors R. Braun and R. Rieger (our institute) for help in editing the manuscript. The skillful technical assistance of Mrs. Astrid Lache is also acknowledged.

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Communicated by C. P. Hollenberg

Received June 24, 1988 / Revised December 2, 1988