

Transformation of the methylotrophic yeast *Hansenula polymorpha* by autonomous replication and integration vectors

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Summary. A high frequency transformation system for the methylotrophic yeast *Hansenula polymorpha* has been developed. This system depends on complementation of isolated uracil auxotrophs by the *URA3* gene of *Saccharomyces cerevisiae*. Maintenance of the uracil prototrophy is based on integration of plasmid YIp5 at random sites within the *H. polymorpha* genome and on autonomously replicating plasmids containing *ARS1* of *S. cerevisiae* or related sequences cloned from the host DNA. The sequence of one autonomously replicating sequence (*HARS1*) from *H. polymorpha* has been determined showing an AT-rich region of 9 bp with some similarity to the consensus sequence of known eukaryotic replication origins. Mitotic loss of autonomously replicating sequences is high; selection for stable uracil prototrophs yields multiple tandem arrangement of the transformed DNA with no detectable loss of the phenotype on non-selective medium. These features offer the possibility for extensive gene expression in *H. polymorpha*.

Key words: Methylotrophic yeasts – Transformation – *ARS* sequence – Plasmid

Introduction

The ability of certain yeast strains to grow on methanol as the sole source of carbon and energy has been well documented. Extensive studies on the metabolism of the C₁ substrate have led to the elucidation of various assimilatory and degradative enzymes, which are regulated by glucose repression and induction by methanol (Tani 1984; Veenhuis et al. 1983; Sahm 1977). Furthermore, growth on methanol is accompanied by the formation of huge peroxisomes, which comprise up to two-thirds of the cellular lumen. Both cytochemical and cell fractionation studies have revealed that the peroxisomes harbour the enzymes of the first oxidation step, namely methanol oxidase (*MOX*), catalase (Fukui et al. 1975; van Dijken et al. 1975; Roggenkamp et al. 1975), and the key assimilatory enzyme, dihydroxyacetone synthase (Goodman 1985). Catalase serves in the removal of H₂O₂, which is formed by the methanol oxidase reaction (Roggenkamp et al. 1974).

Although the metabolism and regulation of methanol utilisation in yeasts is well understood at the enzymatic level, little is known about the genes coding for the respec-

tive enzymes and the molecular mechanisms of gene regulation and peroxisomal development. In a first approach to studying these mechanisms we used in vitro translation experiments, which demonstrated that the biosynthesis of methanol oxidase is regulated at the level of transcription. It was also shown that the primary gene product lacks a cleavable signal sequence at the N-terminus, the presence of which could be expected to mediate transport of the protein into the peroxisomes (Roggenkamp et al. 1984; Roa and Blobel 1983). Furthermore, the *MOX* gene and the gene coding for dihydroxyacetone synthase (*DHAS*) have recently been cloned (Ledeboer et al. 1985; Janowicz et al. 1985; Ellis et al. 1985). In order to achieve an introduction of these cloned genes into *Hansenula polymorpha* a transformation system for this yeast had to be established.

The present paper reports the isolation of auxotrophic mutants of *H. polymorpha* and their complementation by plasmids carrying the corresponding genes from *Saccharomyces cerevisiae*. We show that autonomous replication of these plasmids in *H. polymorpha* can be accomplished both with autonomous replicating sequences from *S. cerevisiae* and with analogous sequences cloned from *H. polymorpha*. Furthermore, transformation by integration of the auxotrophic marker into the *H. polymorpha* genome is demonstrated.

Materials and methods

Strains and media. Bacterial strains used were: *Escherichia coli* 490 (*recA hsdK hsmK met thr thy lacZ*) obtained from G. Hobom and MB 1000 (*hsrK hsmK lac trp pyrF*). The latter strain was originally obtained as a strain carrying the plasmid YIp5 (Stinchcomb et al. 1980), which was cured by acridine orange as described by Miller (1977). For transformation of *S. cerevisiae* strain YNN27 (*trp1-289 ura3-52 gal2*; Stinchcomb et al. 1980) was used. A thermophilic, homothallic strain (ATCC 34438) was used as a wild-type strain of *H. polymorpha*. The mutants LR9, MR7 and NM8 of *H. polymorpha* lack orotidine 5'-phosphate decarboxylase (EC 4.1.1.23) and were designated *ode1* mutants. Strain NM8, in addition to the uracil auxotrophy, has lost the ability to grow on methanol as sole source of carbon and energy but this defect has not been specified until now. Mutants CLK55 and CLK68 which are defective in orotidine 5'-phosphate pyrophosphorylase (EC 2.4.2.10) were designated as *opp1*.

Complex medium for growth of yeasts was YEPD (1% yeast extract, 2% peptone, 2% glucose). Minimal medium for yeasts contained 0.67% YNB (yeast nitrogen base) without amino acids and 2% glucose or synthetic medium (MM) as described (Roggenkamp et al. 1975). Required amino acids or bases were added at a concentration of 20 µg/ml. Growth of *E. coli* cells was in either LB or M9 medium (Maniatis et al. 1982) supplemented with 100 µg/ml ampicillin or 10 µg/ml tetracycline as indicated.

Plasmids. The yeast integrative vector YIp5, containing the *URA3* gene of *S. cerevisiae* as selective marker (Stinchcomb et al. 1980) was used for cloning autonomous replicating sequences from *H. polymorpha*. Further *URA3*-containing yeast plasmids used were: YRp17, bearing an *ARS* sequence (Stinchcomb et al. 1980) and the 2 µm derivative pRB58 (Carlson and Botstein 1982). Plasmids pHARS1 and pHARS2 contain DNA sequences of *H. polymorpha* cloned into the *SalI* site of YIp5 propagating autonomous replication in *H. polymorpha*. Plasmid pHH85 was constructed by insertion of the whole 2 µm DNA (Hollenberg 1982) into the *PstI* site of the ampicillin resistance gene of plasmid YIp5.

Enzymes and chemicals. Restriction enzymes, T4 DNA ligase and DNA polymerase I were purchased from Boehringer (Mannheim, FRG). Ethylmethanesulphonate, nystatin, orotidine 5'-monophosphate, orotidine 5'-monophosphate decarboxylase and 5-phosphorylribose-1-pyrophosphate were products of Sigma (München, FRG). 5-Fluoro-orotic acid was obtained from Pharmacia (Freiburg, FRG) and chemicals used for culture media from Difco (Detroit, MI, USA). All other chemicals were of analytical grade available from commercial sources.

DNA preparations. Minipreparations from *E. coli* were performed as described by Holmes and Quigley (1981). Isolation of plasmid DNA in milligram quantities was performed by the alkaline lysis method (Maniatis et al. 1982).

Yeast minipreparations were done according to Struhl et al. (1979) and isolation of high molecular weight DNA was essentially as described by Janowicz et al. (1985).

Isolation of uracil-requiring mutants of *H. polymorpha*. All procedures were carried out at 30° C instead of 37° C, which is the optimal temperature for growth of this yeast. Yeast cells were mutagenized with 3% ethylmethanesulphonate for 2 h (Fink 1970). The reaction was stopped with 6% sodium thiosulphate (final concentration) and the mixture was incubated for another 10 min. Mutagenized cells were then washed once with H₂O and incubated for 2 days on YEPD or YNB supplemented with uracil, for segregation and enrichment of uracil auxotrophs, followed by a 15 h cultivation on MM without nitrogen source. After 11 h pre-incubation on MM a nystatin enrichment was performed for 1 h at a concentration of 10 µg/ml antibiotic. The treated cells were plated on YNB plates containing 200 µg/ml uracil and 0.8 mg 5-fluoro-orotic acid (Boeke et al. 1984). Usually 10⁶ cells were plated on a single plate. Resistant colonies were picked after 3 days of incubation and replica-plated twice on to YNB plates to establish the auxotrophy. From the auxotrophic mutants Ura⁻ cells were isolated. Alternatively, 1.5 × 10⁶ mutagenized yeast cells were incubated in 1 ml YNB liquid medium supplemented with

200 µg of uracil and 0.8 mg of 5-fluoro-orotic acid. After incubation for 2 days the treated cells were plated on YNB containing uracil, replica-plated twice on to YNB and analysed as described above.

Transformation procedures. Cells of *E. coli* were transformed using the CaCl₂ heat shock method (Maniatis et al. 1982). For transformation of *H. polymorpha* and *S. cerevisiae* the protoplast method was used essentially as described by Beggs (1978). For *H. polymorpha* cells the LiSO₄ method (Ito et al. 1983) and the method described by Klebe et al. (1983), using frozen cells treated with polyethylene glycol, were also employed with the exception that both procedures were performed at 37° C.

Results

Isolation of defined auxotrophic mutants of *H. polymorpha*

The availability of appropriate mutants which can be functionally complemented by a cloned gene is one possible prerequisite for establishing a transformation system. Recently, various auxotrophic mutants of *H. polymorpha* have been described, however, the mutations have not been correlated with corresponding *S. cerevisiae* genes (Sanchez and Demain 1977; Gleeson et al. 1984). We have selected mutants of *H. polymorpha* for 5-fluoro-orotic acid resistance. Such resistant mutants have been shown to be uracil auxotrophs affected at the *URA3* or the *URA5* locus in *S. cerevisiae* (F. Lacroute, personal communication). Of about 800 resistant colonies of *H. polymorpha* tested, 57 exhibited a uracil⁻ phenotype. Since *ura3* and *ura5* mutations in *S. cerevisiae* either lack orotidine 5'-decarboxylase or orotidine 5'-phosphate pyrophosphorylase, respectively (Jones and Fink 1982), the uracil auxotrophs of *H. polymorpha* obtained were tested for both enzymatic activities (Liebermann et al. 1955). Mutants affected in one or other of the two enzymes were found (Table 1) and were designated *odc1* and *opp1* mutants, respectively. The *odc1* mutants ex-

Table 1. Activity of orotidine 5'-phosphate decarboxylase and orotidine 5'-phosphate pyrophosphorylase in *Hansenula polymorpha* mutants requiring uracil for growth

Strain/genotype	Reversion rate	Activity (%) ^a	
		Orotidine 5'-phosphate decarboxylase	Orotidine 5'-phosphate pyrophosphorylase
Wild type	—	100	100
LR9/ <i>odc1</i>	<2 × 10 ⁻⁹	<1	106
MR7/ <i>odc1</i>	6 × 10 ⁻⁷	<1	71
NM8/ <i>idc1</i>	3 × 10 ⁻⁸	<1	105
CLK55/ <i>opp1</i>	n.d. ^b	90	<1
CLK68/ <i>opp1</i>	n.d.	82	<1
YNN27/ <i>ura3</i>	n.d.	0	n.d.

Strains were grown in YEPD until late exponential phase. Extraction of cells was performed with glass beads using a Braun homogenizer. Protein was determined by the optical density at 280 nm

^a Expressed as a percentage of wild-type activity

^b Not determined

Table 2. Transformation of uracil-requiring mutants of *Hansenula polymorpha*

Strain	Plasmid	Trans-formation frequency ^a	Stability ^b (%)	Status of transformed DNA
LR9	YRp17	2.2×10^2	1	Autonomous replication
LR9	pHARS1	1.5×10^3	2	Autonomous replication
LR9	pHARS2	4.6×10^2	1.5	Autonomous replication
LR9	YIp5	3 (38) ^c	101	Integration (single copy)
LR9	pRB58	0	—	—
LR9	pHH85	0	—	—
YNN27	YIp5	0	—	—
LR9	pHARS2	—	105	Integration (multiple)

^a Expressed as the total number per μg of DNA. Frozen cells treated with polyethylene glycol were used for transformation (Klebe et al. 1983). Mean values of four experiments are shown.

^b Expressed as the percentage of remaining uracil prototrophs after growth on YEFD for ten generations. Mean values of two experiments are shown.

^c Number in parenthesis indicates the amount of minicolonies containing free YIp5 plasmid.

hibit adequately low reversion frequencies (Table 1) and thus are suitable for transformation by complementation.

During mutant isolation adenine-requiring mutants were also isolated. Although the nystatin enrichment step was specific for uracil-requiring mutants (see Materials and methods), upon plating more than 90% of the total auxotrophs required adenine for growth. Among these, again more than 90% showed pink or red colour similar to that of the *ade1* and *ade2* mutants of *S. cerevisiae* (Jones and Fink 1982).

Transformation of *H. polymorpha* with an *ARS1* vector of *S. cerevisiae*

Initial transformation experiments were based on the hypothesis that transcription initiation signals and autonomously replicating sequences of *S. cerevisiae* would be recognized in *H. polymorpha* cells. Therefore, plasmid YRP17, which bears the *URA3* gene and *ARS1*, was used in order to transform *odc1* mutants LR9 and MR7. Both mutants could be transformed at frequencies of 2–5 per μg of DNA using the protoplast method and 200–300 transformants using the freeze method of Klebe et al. 1983 (Table 2). Use of the LiSO_4 method resulted in 15–20 transformants per μg of DNA. The frequencies of transformation by the freeze method are of the same order of magnitude as those obtained for *S. cerevisiae* transformed with YRP17 but with the protoplast method are lower for *H. polymorpha* by a factor of more than one thousand (data not shown). This discrepancy can be explained by the fact that *H. polymorpha* is an obligatory aerobic yeast. Protoplast regeneration and/or growth of transformants can thus only occur at the top of the soft agar layer.

Transformation of *H. polymorpha* based on autonomous replication of the vector was indicated by two charac-

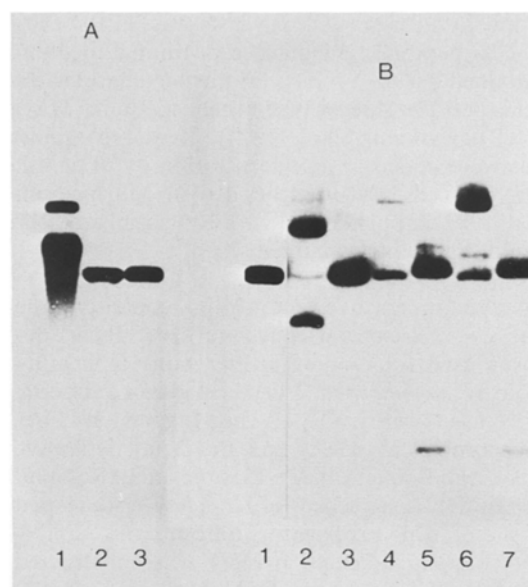


Fig. 1 A, B. Autoradiograms of Southern blots of DNA miniprepations from *Hansenula polymorpha* mutants MR7 or LR9 transformed with YRp17 (A) and pHARS plasmids (B). A Lane 1, transformant MR7-YRp17, undigested DNA; lane 2, as lane 1 but digested with *EcoRI*; lane 3, isolated plasmid YRp17, digested with *EcoRI*. B Lane 1, plasmid pHARS1, digested with *EcoRI*; lane 2, transformant MR7-pHARS1, undigested DNA; lane 3, as lane 2, digested with *EcoRI*; lane 4, plasmid pHARS2, undigested; lane 5, as lane 4, digested with *EcoRI*; lanes 6 and 7, as 4 and 5, but DNA from LR9-pHARS2. For both blots nick-translated YIp5 was used as a hybridization probe.

teristics: (1) by the instability of the uracil⁺ phenotype. After growth of transformants on YEFD for ten generations more than 99% had lost the ability to grow on selective medium (Table 2). (2) Autonomous replication was further ascertained by transforming *E. coli* cells with yeast minilysates and retransformation of *H. polymorpha*. Subsequent Southern analysis showed the presence of plasmid sequences (Fig. 1A). It should be added that the 2 μm DNA replication origin (Broach et al. 1982) obviously does not function in *H. polymorpha* since neither plasmid pRB58 nor pHH85 could be used for transformation.

Isolation of autonomous replicating sequences (*HARS*) from *H. polymorpha*

Chromosomal DNA from *H. polymorpha* was digested with *SalI* and integrated into plasmid YIp5. The ligation mixture was used to transform *E. coli* 490 to ampicillin resistance and the isolated plasmid pool was used to transform *H. polymorpha* mutants LR9 and MR7. A total of 27 prototrophic transformants was obtained. From all of them plasmids could be recovered after transformation of *E. coli* 490 with yeast minilysates. Restriction analysis of the plasmids revealed that most of the inserts showed the same pattern. Two different plasmids, pHARS1 and pHARS2, containing inserts of 0.5 and 2.0 kb respectively, were used for further studies (Fig. 2). Both plasmids transformed *odc1* mutants of *H. polymorpha* with a frequency of about 500–1,500 transformants per μg of DNA using the transformation procedure of Klebe et al. (1983). This frequency is about 2–6 times higher than that obtained with the hetero-

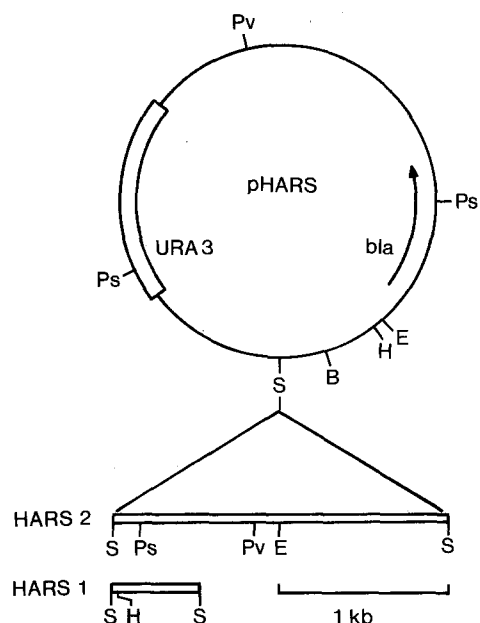


Fig. 2. Alignment of pHARS plasmid. Plasmids are derived by insertion of the indicated *HARS* fragments into the single *SalI* site of YIp5. Restriction sites: E, *EcoRI*; B, *BamHI*; H, *HindIII*; S, *SalI*; Ps, *PstI*; Pv, *PvuII*

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GTCGACTCCC GCGACTCGGC GTTCACTTTC GAGCTATTAT 40
CAACGCCGGA ATACGTCAGA AACAGCCGTG CCCCAGGGAC 80
CAGAAAGCCT ACTGGTGAGT ATGTTCTTTC GTGTGATTTT 120
TCCGAGGATG AGAACGACGA TAACGAGCAC AACTCGGAGT 160
CGGAGGACAC GCTTATTGCG TTGAACGCAG CCACATCAGC 200
AGGCTGTCAA GACTGAGTAT GGCCACAGAG CTGGATTCTC 240
GGCCTCATAC TCAAGACGTT AGTAACTCC GTCTGCCAGA 280
AATTGCTGAC GAGGATGTAI AATAATAGAT GAATTACGAA 320
CAATTGTAGT TCAAAAAAAT TTAGTAACAA TATTGTCTAG 360
ATGACAGATG TGCTGAAACC AGTGAACCTC AATAAACCCAC 400
TCACCGCTAC CCAAGAGAAA CAGATCAGAG TGCTAGGGCC 440
TTGTTTCAGA GTACTACAAC GTTTACCAGA AGCTTAGGCA 480
AGTTCTCAAA CGCGGGTTTG TCGAC

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Fig. 3. Nucleotide sequence of *HARS1* from *Hansenula polymorpha*. The original *SalI* fragment was cloned into the phage M13 derivatives mp8 and mp9 (Messing and Vieira 1982) and sequenced by the dideoxy chain termination method (Sanger et al. 1977; Sanger et al. 1980). The orientation is 5' to 3', left to right. The AT sequence of nine nucleotides with an alphabetic symmetry is underlined

logous *ARS1* of *S. cerevisiae* (Table 2). The stability of the *HARS* plasmids in transformants is slightly higher than the *ARS1* plasmid (Table 2). Southern analysis of the *H. polymorpha* transformants after retransformation with pHARS1 and pHARS2 recovered from *E. coli* 490 shows the expected plasmid bands and thus excludes integration of the *URA3* gene as a cause of the uracil prototrophy (Fig. 1B). Therefore, we conclude that the *HARS* sequences, like *ARS1*, allow autonomous replication in *H. polymorpha*.

DNA sequence of *HARS1*

The nucleotide sequence of the *SalI* fragment bearing *HARS1* was determined. The fragment comprises 505 bp (Fig. 3) and does not contain an open reading frame. There

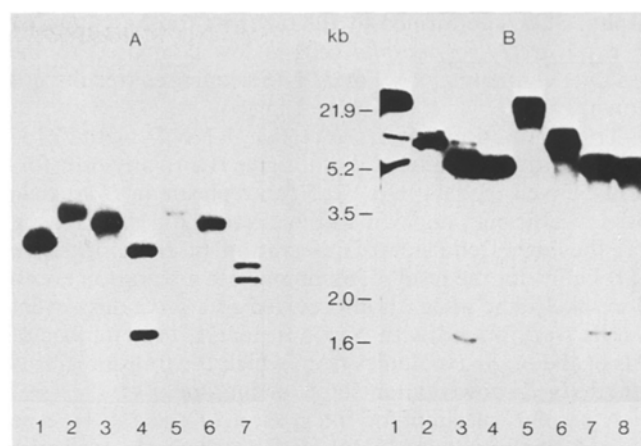


Fig. 4A, B. Autoradiogram of Southern blots of DNA from *Hansenula polymorpha* mutant LR9 transformed by integration of plasmids YIp5 (A) and pHARS2 (B). A Lane 1, authentic plasmid YIp5, digested with *EcoRI*; lanes 2-4 and lanes 5-7 show two different transformants. Lanes 2, 5, undigested DNA; lanes 3, 6, digested with *EcoRI*; lanes 4, 7, digested with *PvuII*. B Lanes 1-4, transformant LR9-pHARS2 (free plasmid); lanes 5-8, transformant LR9-pHARS2 (multiple integrant); lanes 1, 5, undigested DNA; lanes 2, 6, digested with *HindIII*; lanes 3, 7, digested with *EcoRI*; lanes 4, 8, digested with *SalI*. For both blots nick-translated YIp5 was used as a hybridization probe

is a sequence of nine nucleotides at position 299-307 which share some common features with the consensus sequence proposed for autonomously replicating sequences from *S. cerevisiae* (Broach et al. 1982; Kearsley 1984), that is an AT-rich sequence of similar size with an alphabetic symmetry followed by an AT-rich region towards the 3' end.

Transformation of *H. polymorpha* by integration of the *URA3* gene from *S. cerevisiae*

The *URA3* gene of *S. cerevisiae* shows no detectable homology with the *ODC* gene in *H. polymorpha*, as revealed by Southern hybridisation of nick-translated YIp5 plasmid DNA to chromosomal DNA of *H. polymorpha* (not shown). Therefore, low frequency integration of the *URA3* gene into the *H. polymorpha* genome had to be anticipated. Transformation of mutant LR9 with the integrative vector YIp5 resulted in 30-40 colonies per μ g of DNA on YNB plates using the polyethylene glycol method, whereas no transformants were obtained in the control experiment using YIp5 to transform *S. cerevisiae* mutant YNN27 (Table 2). Analysis of 38 transformants revealed 4 stable integrants after growth on non-selective medium. The integration event in two transformants was further demonstrated by Southern analysis (Fig. 4A). Restriction of DNA by *PvuII* revealed two hybridizing bands of smaller size than the *PvuII*-linearized plasmid YIp5, which is in line with a single copy integration for both transformants (Fig. 4A, lanes 4, 7). The rest of the transformants formed only mini-colonies and were very unstable when grown on non-selective medium. They contained the authentic YIp5 plasmid as shown in *E. coli* after transformation with yeast minilyesates (data not shown). We conclude that the YIp5 plasmid in *H. polymorpha*, in contrast to *S. cerevisiae*, can be replicated with a very low efficiency leading to the uracil proto-

trophy. This is confirmed by the fact that the copy number of YIp5 in *H. polymorpha* cells is low compared to the plasmids containing *ARS* or *HARS* sequences (results not shown).

Transformation of *S. cerevisiae* YNN27 with YIp5 under the same conditions did not give rise to any transformants. Given the fact that YIp5 can replicate in *Hansenula* with low efficiency and can lead to microcolonies, we reason that the high frequency of integration in *H. polymorpha* is probably not the result of an immediate integration event. More likely, the integration occurred as a secondary event in cells transformed with YIp5. In fact, a low number of cells of the original colonies from which the four integrants were derived showed an unstable phenotype.

A second selection for integration of the *URA3* gene into the chromosomal DNA of *H. polymorpha* was performed by enrichment of stable *Ura*⁺ transformants from transformants carrying the autonomously replicating plasmid pHARS2. Transformants were grown in liquid YEPD to a density of 10⁹ cells per ml. An aliquot containing 5 × 10⁶ cells was used to inoculate 100 ml of fresh medium and was grown to a cell density of 10⁹ per ml. The procedure was repeated until about 100 generations had been reached. Since the reversion rate of mutant LR9 is 2 × 10⁻⁹ and the frequency of plasmid loss per 10 generations is 98% for pHARS2 transformants, the predominant part of the *Ura*⁺ cells after 100 generations should be integrants. The *Ura*⁺ colonies tested were all shown to maintain a stable *Ura*⁺ phenotype indicating integration of the *URA3* gene. This was further verified by Southern blot analysis (Fig. 4B). In addition, these data indicate that the integration frequency is higher than 5 × 10⁻⁶.

The intensity of the Southern hybridization of the integrated DNA shown in Fig. 4B suggests multiple integrations of plasmid pHARS2 with the same high copy number as is present in transformants containing the free plasmid. Restriction analysis of DNA from minilysates shows fragments of the same size as the original linearized plasmid indicating multiple tandem integrations (Fig. 4B, lanes 6–8). It should be mentioned that transformation of *E. coli* cells both from a *recA*⁻ and a *recA*⁺ strain can be achieved at low numbers with DNA minipreparations from integrants. Such *E. coli* transformants contain the authentic plasmid (not shown), which is probably generated in the yeast transformants by recombination.

However, the Southern analysis alone cannot prove multiple integration unequivocally. A multimeric form of free plasmid pHARS2 could also be proposed, although this interpretation would not be in line with the high stability of the plasmid and the fact that transformation with yeast minilysates shows the same frequency in a *recA*⁻ and a *racA*⁺ strain of *E. coli*.

Estimation of plasmid copy number in *H. polymorpha* transformants

The copy numbers of plasmids replicating autonomously in *H. polymorpha* either by *ARS* sequences or by *HARS* sequences and that of integrants were estimated by Southern blot analysis (Fig. 5). For comparison, plasmid YRp17 in *S. cerevisiae* (Fig. 5, lanes 6, 7), which has a copy number of 5–10 per cell (Struhl et al. 1979), and the high copy number plasmid pRB58 in *S. cerevisiae* (Fig. 5, lanes 4, 5), with about 30–40 copies per cell (Erhart 1983), were used.

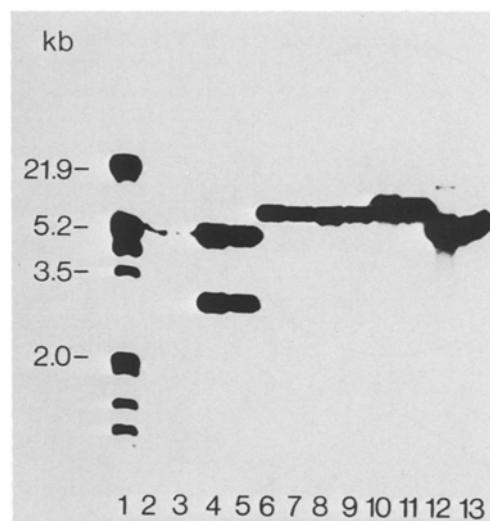


Fig. 5. Estimation of copy number by Southern hybridization of DNA from *Hansenula polymorpha* transformants. Aliquots of 8 and 16 μ l were electrophoresed. Lane 1, phage DNA digested with *Hind*III and *Eco*RI; lanes 2, 3, transformant of *Kluyveromyces lactis* containing two copies of integrated plasmid, digested with *Hind*III (M. Reynen, K. Breunig and C.P. Hollenberg, unpublished results); lanes 4–7, YNN27, transformed with pRB58 (4, 5) and YRp17 (6, 7), digested with *Eco*RI; lanes 8, 9, LR9 transformed with YRp17, digested with *Eco*RI; lanes 10, 11, LR9 transformed with pHARS2, digested with *Hind*III; lanes 12, 13, LR9 transformed with pHARS1 digested with *Eco*RI

Table 3. Activity of β -lactamase in various transformants of *Hansenula polymorpha*

Plasmid	Status of transformed DNA	Specific activity of β -lactamase ^a	Copy number
—	—	0	—
YRp17	Autonomous replication	3.8×10^{-3}	5
pHARS1	Autonomous replication	3.2×10^{-2}	40
pHARS2	Autonomous replication	2.0×10^{-2}	25
YIp5	Integration (single copy)	8.0×10^{-4}	1
pHARS2	Integration (multiple)	6×10^{-2}	75

^a Expressed as μ mol/min per mg protein. Protein was determined by the method of Lowry et al. 1951

A *Kluyveromyces lactis* transformant carrying two integrated copies of pBR322 was used as a control (Fig. 5, lanes 2, 3). The intensity of hybridisation in the autoradiogram shows that plasmid YRp17 in *H. polymorpha* (Fig. 5, lanes 8, 9) has practically the same copy number of 5–10 as in *S. cerevisiae*, whereas plasmids pHARS1 and pHARS2 show a copy number which is in the range of 30–40 copies per cell like pRB58 in *S. cerevisiae* (Fig. 5, lanes 10–13).

A more precise estimation of copy number was performed by measuring β -lactamase activity conferred by the *bla* gene in *H. polymorpha* transformants. A transformant carrying a single integrated copy of YIp5 was used as a standard. Comparison of specific β -lactamase activities of the various transformants indicates copy numbers of 5 for YRp17 and of 40 and 25 for pHARS1 and pHARS2, re-

spectively (Table 3). The multiple integration of plasmid pHARS1 gives up to 75 copies per cell (Table 3).

Discussion

This report has demonstrated transformation of the methylotrophic yeast *H. polymorpha* by complementation of an orotidine 5'-decarboxylase mutation with the *URA3* gene of *S. cerevisiae*. We have shown that both integration of the *URA3* gene into the *H. polymorpha* genome and autonomous replication can establish uracil prototrophy and that autonomous replication is maintained both by *ARS1* from *S. cerevisiae* and related sequences isolated from *H. polymorpha*. Although the heterologous *ARS1* functions in *H. polymorpha*, neither *HARS1* nor *HARS2* shows autonomous replication in *S. cerevisiae*. In addition, cloned sequences of *H. polymorpha* selected as replication origins in *S. cerevisiae* do not function in *H. polymorpha* (our unpublished results).

Besides *S. cerevisiae*, a variety of yeast species have been transformed successfully, including *Schizosaccharomyces pombe* (Beach and Nurse 1981; Sakaguchi and Yamamoto 1982), *Kluyveromyces lactis* and *fragilis* (Das and Hollenberg 1982; Sreekrishna et al. 1984; Das et al. 1984) and *Candida maltosa* as well as *Pichia guilliermondii* (Kunze et al. 1985). Transformation by autonomous replication by an *ARS* originating from the host has been described for *K. lactis* (Das and Hollenberg 1982) and *Schizosaccharomyces pombe* (Sakaguchi and Yamamoto 1982). For the other yeast species heterologous origins of replication have been used.

The transformation frequency of *H. polymorpha* cells is high using *HARS* sequences for autonomous replication. Values can be at least as high as those obtained in *S. cerevisiae* with *ARS1* plasmids. The same holds true for mitotic loss of these plasmids; the frequency of plasmid loss is as high as with *ARS* sequences in *S. cerevisiae* (Struhl et al. 1979).

The occurrence of stable phenotypes with multi-copy tandem arrangements of plasmid sequences shown for *H. polymorpha* seems to be a rather specific feature in this yeast, although smaller polymeric forms have also been described for plasmids that transform *S. pombe* with high frequency (Sakaguchi and Yamamoto 1982). The determinants and mechanisms of such polymerization events remain to be investigated. Especially surprising is the fact that the extensive increase of plasmid number in *H. polymorpha* is observed after growth on non-selective medium. Such gene amplification seems to be a promising tool for the efficient expression of homologous and heterologous genes in this yeast.

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