MOLECULAR AND CELLULAR BIOLOGY, Dec. 1985, p. 3376–3385
0270-7306/85/123376-10$02.00/0

Vol. 5, No. 12

Pichia pastoris as a Host System for Transformations

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Received 11 June 1985/Accepted 11 September 1985

We developed a methylotrophic yeast, *Pichia pastoris*, as a host for DNA transformations. The system is based on an auxotrophic mutant host of *P. pastoris* which is defective in histidinol dehydrogenase. As a selectable marker, we isolated and characterized the *P. pastoris HIS4* gene. Plasmid vectors which contained either the *P. pastoris* or the *Saccharomyces cerevisiae HIS4* gene transformed the *P. pastoris* mutant host. DNA transfer was accomplished by a modified version of the spheroplast generation ($CaCl_2$-polyethylene glycol)-fusion procedure developed for *S. cerevisiae*. In addition, we report the isolation and characterization of *P. pastoris* DNA fragments with autonomous replication sequence activity. Two fragments, PARS1 and PARS2, when present on plasmids increased transformation frequencies to 10^5/μg and maintained the plasmids as autonomous elements in *P. pastoris* cells.

Methylotrophic yeasts such as *Pichia pastoris* are of both academic and industrial interest. One reason for this interest is to study the regulation of gene expression by methanol. In these yeasts, expression of the gene which encodes alcohol oxidase (AO), the first enzyme in the methanol utilization pathway, is tightly regulated (34, 35). When the yeasts are grown on glucose or ethanol, AO is not detectable in the cells. However, when the yeasts are grown on methanol, AO can be up to 35% of the total cellular protein (12, 38, 49). Recently, it was shown that control of AO expression in *P. pastoris* is substantially transcriptional (17). A second point of interest in methylotrophic yeasts is the mechanism by which enzymes such as AO and catalase are compartmentalized in peroxisomes (18). Peroxisomal packaging of AO does not appear to involve a processed amino-terminal signal sequence (17). A third interest, which is primarily industrial, is in the use of methylotrophic yeasts for single-cell protein production (1, 11, 41).

The study of methylotrophic yeasts at the molecular level has been hampered by the inability to transfer and maintain DNA sequences of interest in the yeasts. The missing link, a transformation system, is essential to correlate DNA sequence structure with function and to express foreign genes in the organism. To date, yeast transformation systems have been reported for *Saccharomyces cerevisiae* (3, 22), *Schizosaccharomyces pombe* (2), *Kluyveromyces lactis* (14), and *Kluyveromyces fragilis* (15). This is the first report of a transformation system in methylotrophic yeasts.

In this paper we describe an efficient transformation system for *P. pastoris*. The system is based on a histidinol dehydrogenase-defective mutant host of *P. pastoris* and a modified version of the spheroplast fusion-gene transfer procedure developed by Hinnen et al. (22) for *S. cerevisiae*. As a selectable marker, we isolated and characterized the *P. pastoris HIS4* gene. Plasmids which contained either the *Pichia* or the *Saccharomyces HIS4* gene complemented the mutant *P. pastoris* host defect. In addition, we describe the isolation and characterization of *P. pastoris* DNA fragments with autonomous replication sequence (ARS) activity. Plasmids which contain a *Pichia* ARS (PARS) fragment transform at frequencies of about 10^5/μg and are maintained in *P. pastoris* cells as extrachromosomal elements.

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MATERIALS AND METHODS

Strains, plasmids, and media. The wild-type *P. pastoris* strain used was NRRL Y-11430 (Northern Regional Research Center, Peoria, Ill.). *P. pastoris* GS115 (*his4*) was generated by nitrosoguanidine mutagenesis of NRRL Y-11430 and was a gift from George Sperl, Phillips Petroleum Co. *S. cerevisiae* 5799-4D (**a** *his4-260 his4-39*) and 6657-9B (**a** *his4-34 leu2-3 leu2-112* Can^r) were gifts from G. Fink, Massachusetts Institute of Technology. *Escherichia coli* 848 (F^- *met thi gal* T_1R $\phi 80^S$ *hsdR hsdM*$^+$) was used in all experiments which required a bacterial host.

YIp25, which consists of the *S. cerevisiae HIS4* gene on a 9.4-kilobase (kb) *Pst*I fragment inserted at the *Pst*I site of pBR322 (16), was the source of the *S. cerevisiae HIS4* gene fragments and was a gift from G. Fink. YEp13 (10), pBR322 (6), and pBR325 (5) have been described previously.

The complete medium (YPD) and minimal medium (SD) for growing *S. cerevisiae* are described in Sherman et al. (40). *P. pastoris* was grown in YPD or IMG. IMG, a minimal medium, consists of the following: IM_1 salts at a final concentration of 36.7 mM KH_2PO_4, 22.7 mM $(NH_4)_2SO_4$, 2.0 mM $MgSO_4 \cdot 7H_2O$, 6.7 mM KCl, and 0.7 mM $CaCl_2 \cdot 2H_2O$, prepared as a 10× stock and autoclaved; trace salts at a final concentration of 0.2 μM $CuSO_4 \cdot 5H_2O$, 1.25 μM KI, 4.5 μM $MnSO_4 \cdot H_2O$, 2.0 μM $NaMoO_4 \cdot 2H_2O$, 0.75 μM H_3BO_3, 17.5 μM $ZnSO_4 \cdot 7H_2O$, 44.5 μM $FeCl_3 \cdot 6H_2O$, prepared as a 400× stock and filter sterilized; 0.4 μg of biotin per ml; and 2% dextrose. *E. coli* was cultured in either LB medium (30) or 2B medium (0.2% NH_4Cl, 0.6% KH_2PO_4, 1.2% Na_2HPO_4, 0.013% $MgSO_4 \cdot 7H_2O$, 0.074% $CaCl_2 \cdot 2H_2O$, 1 μg of thiamine per ml, 0.4% dextrose), supplemented with 100 μg of tryptophan per ml and 0.2% Casamino Acids (Difco Laboratories, Detroit, Mich.).

DNA isolation. Large-scale preparations of DNA from both *P. pastoris* and *S. cerevisiae* were done by a modification of the method of Cryer et al. (13). Yeast cells were grown in 100 ml of minimal medium to an A_{600} of 1 to 2 and harvested by centrifugation at 2,000 × *g* for 5 min. The cells were washed successively with 5 ml of H_2O, SED (1 M sorbitol, 25 mM EDTA, 50 mM dithiothreitol), and 1 M sorbitol and suspended in 5 ml of 1 M sorbitol–0.1 M Tris hydrochloride (pH 7.0). The cells were mixed with 50 to 100 μl of a 4-mg/ml solution of Zymolyase 60000 (Miles, Inc., Elkhart, Ind.) and incubated at 30°C for 1 h to digest the cell

walls. The spheroplast preparation was then centrifuged at 1,000 × *g* for 5 to 10 min and suspended in lysis buffer (0.1% sodium dodecyl sulfate, 10 mM Tris hydrochloride [pH 7.4], 5 mM EDTA, 50 mM NaCl). Proteinase K (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and RNase A (Sigma Chemical Co., St. Louis, Mo.) were each added to a concentration of 100 μg/ml, and the mixtures were incubated at 37°C for 30 min. DNA was deproteinized by gently mixing the preparation with an equal volume of chloroform containing isoamyl alcohol (24:1, vol/vol), and the phases were separated by centrifugation at 12,000 × *g* for 20 min. The upper aqueous phase was drawn off into a fresh tube and extracted with an equal volume of PCA (phenol-chloroform-isoamyl alcohol, 25:24:1, vol/vol/vol). The phases were separated as before, and the top phase was removed to a tube containing 2 to 3 volumes of cold (−20°C) 100% ethanol. The sample was gently mixed, and DNA was collected by spooling onto a plastic rod. The DNA was immediately dissolved in 1 ml of TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA) and dialyzed overnight against 100 volumes of 4°C TE buffer. Small-scale yeast DNA preparations were by the procedure described in Sherman et al. (40).

E. coli cultures for large-scale (>0.5-liter) plasmid preparations were grown at 37°C with shaking in 2B medium supplemented as described above and with the appropriate antibiotic. Strains which contained pBR325-derived plasmids were inoculated into the supplemented 2B medium at a starting A_{550} of 0.01 to 0.05 and incubated with shaking at 37°C for 20 to 24 h before harvesting. For strains which contained pBR322-derived plasmids, cultures were grown to an A_{550} of 0.7, at which time chloramphenicol was added to a concentration of 100 μg/ml, and harvested approximately 15 h later. For small-scale rapid plasmid isolations, 2-ml cultures in the supplemented 2B medium with antibiotic were grown overnight at 37°C with shaking and harvested by centrifugation in 1.5-ml microcentrifuge tubes. Plasmids from all preparations were isolated by the alkaline lysis method described by Birnboim and Doly (4).

DNA methods. Restriction enzymes were obtained from New England BioLabs, Inc. (Beverly, Mass.) and Bethesda Research Laboratories, Inc., (Gaithersburg, Md.), and digestions were performed as recommended by the suppliers. Restriction mappings were carried out by comparing parallel digestions of plasmids with and without insert DNA. Restriction fragments were purified by electroelution from agarose gels into Whatman 3MM paper strips backed by dialysis tubing as described by Girvitz et al. (19). The fragments were recovered from the paper and tubing by three to four washings with 0.1- to 0.2-ml volumes of a solution which contained 0.1 M NaCl, 50 mM Tris hydrochloride (pH 8.0), and 1 mM EDTA. Finally, the fragments were extracted with PCA, precipitated with ethanol, and dissolved in a small volume of TE buffer.

For the *P. pastoris* DNA-YEp13 library construction, 100 μg of YEp13 was digested to completion with *Bam*HI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim) as described by Maniatis et al. (28). A 100-μg sample of wild-type *P. pastoris* DNA was partially digested with 10 U of *Sau*3AI by incubation for 5 min at 37°C in a total volume of 1 ml. Fragments of 5 to 20 kb were size selected by centrifugation through 5 to 20% sucrose gradients as described by Maniatis et al. (28). A 1-μg sample of the vector and 2 μg of the *Pichia Sau*3AI fragments were mixed with 20 U of T4 DNA ligase (Bethesda Research Laboratories) in a total volume of 200 μl and incubated overnight at 4°C. The ligated DNAs were transformed into *E. coli* by a modification of the $CaCl_2$ method of Mandel and Higa (27). For this, the entire ligation reaction mix was added to 2 ml of competent *E. coli* 848 cells and then incubated on ice for 15 min. The mixture was shifted to 37°C for 5 min, after which 40 ml of LB medium was added, and the 37°C incubation was continued for 1 h. Ampicillin was then added to a concentration of 100 μg/ml, and the incubation continued for a second hour. Finally, the cells were centrifuged for 10 min at 3,000 × *g*, resuspended in 1 ml of fresh LB medium, and spread in equal aliquots on 10 LB agar plates containing 100 μg of ampicillin per ml. The approximately 50,000 colonies which resulted were scraped from the plates, and a portion of the cells was inoculated into 500 ml of the supplemented 2B medium at a starting A_{550} of 0.1. The culture was grown and plasmids were extracted as described above. From the colonies which were pooled for the library, 96 of 100 tested were tetracycline sensitive and 7 of 10 examined contained plasmids with insert DNA.

The PARS selection library was constructed as described above except that *Pichia Taq*I partial fragments of 5 to 10 kb were isolated and ligated with *Cla*I-cut, dephosphorylated pYJ8Δ*Cla*.

DNA hybridizations were performed by the method of Southern (42) as described by Maniatis et al. (28). For transfer of large or supercoiled DNA molecules to nitrocellulose, DNA was first partially hydrolyzed by soaking agarose gels in 0.25 M HCl for 10 min before alkali denaturation (50). The hybridization of labeled fragments from the *S. cerevisiae HIS4* gene to *P. pastoris* DNA was performed in the presence of 50% formamide, 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 0.1% sodium dodecyl sulfate, 1 mM EDTA, and 100 μg of denatured herring sperm DNA per ml at 42°C. Post-hybridization washes were in 2× SSC–1 mM EDTA–0.1% sodium dodecyl sulfate–0.1% sodium PP_i at 55°C. DNA was ^{32}P labeled by the method of Rigby et al. (33).

DNA was sequenced by the dideoxynucleotide chain termination method of Sanger et al. (39).

Yeast transformations. *S. cerevisiae* transformations were performed by the spheroplast generation method of Hinnen et al. (22) or the intact-cell method of Ito et al. (23).

P. pastoris transformations were performed by the method of Hinnen et al. (22) with the following modifications. A fresh colony of GS115 was inoculated into 10 ml of YPD and grown with shaking at 30°C overnight. The afternoon of the next day, a 10- to 100-μl sample of the culture was added to 100 ml of fresh YPD and grown overnight as before to an A_{600} of 0.2. The cells were harvested by centrifugation at 2,000 × *g* for 5 min and washed successively with 10 ml of H_2O, 10 ml of freshly prepared SED, and twice with 10 ml of 1 M sorbitol and then suspended in 10 ml of SCE buffer (1 M sorbitol, 0.1 M sodium citrate [pH 5.8], 10 mM EDTA). The cells were then mixed with 10 μl of a 4-mg/ml solution of Zymolyase 60000 and incubated at 30°C for 30 min. The spheroplasts were washed twice in 10 ml of 1 M sorbitol and once in 10 ml of CaS (10 mM $CaCl_2$, 1 M sorbitol) and suspended in 0.6 ml of CaS. Samples of 100 μl containing 1×10^7 to 3×10^7 regeneratable spheroplasts were incubated with plasmid DNA and 5 μg of sonicated *E. coli* DNA for 20 min at room temperature. Then, 1 ml of 20% polyethylene glycol 3350 (Fisher Scientific Co., Pittsburgh, Pa.) in 10 mM Tris hydrochloride (pH 7.4)–10 mM $CaCl_2$ was added. The samples were incubated for an additional 15 min at room temperature and then centrifuged at 1,000 × *g* for 10 min. The spheroplasts were suspended in 150 μl of SOS

medium (1 M sorbitol, 0.3× YPD, 10 mM $CaCl_2$), incubated at room temperature for 30 min, and then diluted to a total volume of 1 ml with 1 M sorbitol. Finally, portions were added to 10 ml of regeneration agar (3% agar, 1 M sorbitol, SD medium, 0.4 μg of biotin per ml) and poured on top of a base plate containing 10 ml of the same regeneration agar medium. Plates were incubated for 3 to 5 days at 30°C.

Analysis of *P. pastoris* transformants for stability, copy number, and state of plasmid. A determination of the stability of plasmids was performed by growing transformed *P. pastoris* cultures logarithmically in IMG medium at 30°C with shaking for at least 50 generations. At points approximating 0, 10, 25, and 50 generations, samples of cultures were removed for analysis. One portion of each sample was sonicated, diluted, and spread on four SD medium agar plates, two without and two with 20 μg of histidine per ml. The sonication step was necessary to separate *P. pastoris* cells, which tend to grow in clumps. For sonication, samples of cells were diluted to an A_{600} of 0.1 and sonicated for 10 s with a Sonifier Cell Disrupter 350 (Branson Sonic Power Co., Danbury, Conn.) at power setting 4, a treatment which is sufficient to separate cells but not to reduce cell viability. A second portion of each sample was inoculated into IMG medium containing histidine. After about 10 generations (approximately 24 h), cultures were sampled and assayed for the His phenotype as described above. A third portion of each sample was a source of DNA for Southern blot analysis for the state of the plasmid.

An average plasmid copy number per *P. pastoris* cell was derived from the ratio of the amount of the genomic copy of the *P. pastoris HIS4* gene to that of a plasmid-borne *HIS4* gene. For strains which contained plasmids with the *Pichia HIS4* gene, DNAs were extracted, digested with restriction endonucleases, electrophoresed into an agarose gel, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled 2.7-kb *Bgl*II fragment containing the *Pichia HIS4* gene. After posthybridization washing, a series of X-ray films were exposed to the filter for specific lengths of time and scanned on a Beckman DU-8B spectrophotometer which was programmed with a Compuset Module for slab gels. For *P. pastoris* strains transformed with plasmids which contained the *Saccharomyces HIS4* gene, the procedure just described was performed with the following modifications: (i) 10 ng of restricted pYJ28, a plasmid which contains copies of both the *Saccharomyces* and the *Pichia HIS4* genes, was electrophoresed into the gels with the yeast DNAs, and (ii) the filter-bound DNAs were hybridized to a mixture of two ^{32}P-labeled probes, the *Pichia HIS4* fragment described above and a 2.7-kb *Sal*I fragment from pYA3 which contained a major portion of the *Saccharomyces HIS4* gene (plus 89 base pairs (bp) of pBR322). The ratio of the intensity of the band corresponding to the *Pichia* genomic *HIS4* fragment to that of the band from the plasmid-borne *Saccharomyces HIS4* fragment band was determined as before and then adjusted by a factor derived from scans of the pYJ28-containing lanes which corrected for the difference in the relative intensity of the *Saccharomyces* and *Pichia HIS4* bands.

RESULTS

Isolation of a *his4* mutant strain and the *HIS4* gene of *P. pastoris*. As first steps in developing a transformation system for *P. pastoris*, an auxotrophic host and the *P. pastoris* gene capable of complementing the mutant host defect were isolated. To identify a mutant strain with a specific enzymatic defect, we examined a collection of histidine-requiring strains of *P. pastoris* for ATP phosphoribosyltransferase, imidazoleglycerol-phosphate-dehydratase, histidinol-phosphate phosphatase, and histidinol dehydrogenase activities (29). One His^- auxotroph, GS115, was found to have no detectable histidinol dehydrogenase activity and possessed a reversion frequency to histidine prototrophy of less than 10^{-8}.

DNA fragments which contained the *Pichia HIS4* gene were isolated from a *Pichia* DNA library by their ability to complement *S. cerevisiae his4*$^-$ strains. The library was composed of 5- to 20-kb *Sau*3AI partial digestion fragments of wild-type *Pichia* DNA inserted into the *Bam*HI site of the *S. cerevisiae-E. coli* shuttle vector YEp13. Spheroplasts of 5799-4D, a *his4ABC*$^-$ strain of *S. cerevisiae*, were mixed with the *Pichia* DNA library and allowed to regenerate in a medium deficient in histidine. The transformation resulted in about 1×10^3 prototrophic yeast colonies from a population of 5×10^7 total regeneratable spheroplasts. Total yeast DNA was extracted from 20 of the His^+ colonies and transformed into *E. coli*. Seventeen of the yeast DNA preparations produced ampicillin-resistant colonies, and each contained plasmids comprised of YEp13 plus insert DNA. Eleven of these plasmids were further examined by transformation into 6657-9B, a *his4AB*$^-$ *leu2*$^-$ strain of *S. cerevisiae*. Eight plasmids transformed this strain to histidine prototrophy at a frequency indistinguishable from controls in which leucine prototrophy was selected. The remaining three plasmids did not produce His^+ transformants. To confirm that the eight His^+ transforming plasmids contained the *Pichia HIS4* gene and not a DNA fragment with suppressor activity, restriction digests of the plasmids were hybridized to a labeled DNA fragment containing a large portion of the *S. cerevisiae HIS4* gene and washed at low stringency. Each of the eight plasmids which complemented the *his4*$^-$ *S. cerevisiae* strains contained sequences which hybridized to the *S. cerevisiae HIS4* gene, while YEp13 and the three noncomplementing plasmids did not.

Transformation of *P. pastoris*. Each of the *Pichia HIS4* gene-YEp13 recombinants described above transformed *P. pastoris* GS115 at a high frequency. One of these plasmids, pYA4, which contained the *Pichia HIS4* gene on the smallest fragment, a 6.0-kb *Sau*3AI partial fragment, was selected for further analysis. A restriction endonuclease cleavage site map of pYA4 is shown in Fig. 1, and the results of analysis of pYA4 transformants are shown in Table 1. *Pichia* spheroplasts were transformed at a high frequency, which suggested that the plasmid exists as an autonomous element in *Pichia* cells. Evidence for the autonomous nature of pYA4 in *P. pastoris* was provided by three observations. First, the plasmid could be rescued from *Pichia* cells by transformation of *E. coli* cells with the yeast DNA (data not presented). Second, the His^+ phenotype was lost from cells grown in nonselective medium (Table 1). Third, a typical plasmid pattern could be observed in Southern blots in which unrestricted DNAs from pYA4 transformants were hybridized to labeled YEp13. Figure 2 shows the result of one such Southern blot experiment. Lane 2 of the figure contains DNA from a culture which was sampled after about 10 generations and shows a hybridization pattern which is indistinguishable from that of the *E. coli*-extracted pYA4 pattern in lanes 1 and 7. However, with DNA samples from cells of the same culture which had grown for a total of 25 or more generations (lanes 3 and 4), the YEp13 probe hybridized at the position of the unrestricted chromosomal DNAs. Thus, the eventual fate of pYA4 was integration into the *Pichia* genome.

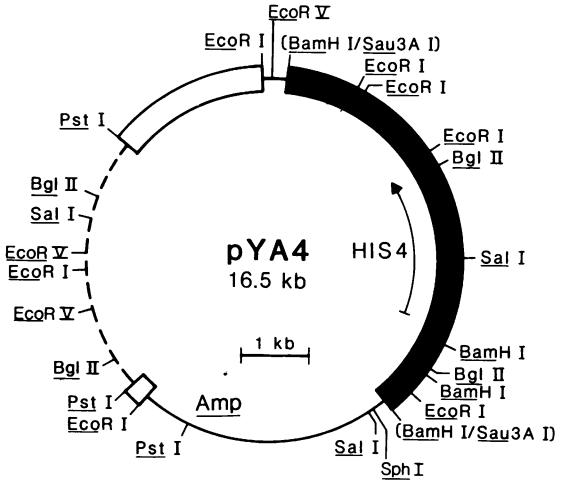


FIG. 1. Restriction enzyme map of pYA4. The plasmid contains sequences from the following sources: pBR322, thin line; *S. cerevisiae LEU2* gene, dashed line; *S. cerevisiae* 2μm plasmid, open boxes; *P. pastoris HIS4* gene, thick line. The position and direction of transcription of the *HIS4* gene are shown by the arrow. Amp, Ampicillin.

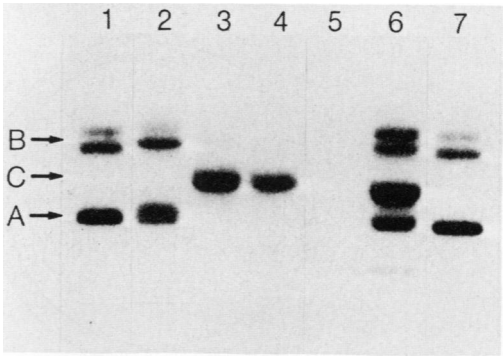


FIG. 2. Southern blot hybridization of yeast cellular DNAs with ^{32}P-labeled YEp13. Lanes 1 and 7 each contain 10 ng of unrestricted pYA4 from *E. coli* cells. Lanes 2, 3, and 4 contain 1 μg of unrestricted DNA from pYA4-transformed GS115 cells which had been grown in selective medium for 10, 25, and 50 generations, respectively. Lane 5 contains 1 μg of unrestricted DNA from untransformed GS115. Lane 6 contains 1 μg of unrestricted DNA from *S. cerevisiae* cells transformed with pYA4. The arrows mark the positions of the following DNA species: A, supercoiled plasmid; B, nicked circular and supercoiled dimer plasmid; C, chromosomal. Under the electrophoretic conditions used in the experiment, nicked circular and supercoiled dimer plasmid species of pYA4 migrate at a slower rate than much larger linear chromosomal DNAs.

Vectors which contained the *S. cerevisiae HIS4* gene as the selectable marker could transform GS115 as well. Figure 3 is a restriction site map of pYA2, a vector composed of a 9.4-kb *Pst*I fragment containing the *S. cerevisiae HIS4* gene (16, 21) inserted into the *Pst*I site of pBR325. pYA2 transformed GS115 at a high frequency and was maintained as an autonomous element for over 50 generations in selective medium (Table 1). The vector is not autonomous in *S. cerevisiae* (22; J. Cregg, unpublished observation). Further experiments in which portions of pYA2 were subcloned and transformed into *Pichia* cells indicated that the strongest ARS is located somewhere in the *S. cerevisiae* sequences flanking the *HIS4* gene, that a second weak ARS is in the *S. cerevisiae HIS4* gene itself, and that pBR325 has no detectable ARS activity (data not shown).

Since pYA2 is autonomous in GS115 cells for long periods of growth in selective medium, it would appear that the *S. cerevisiae*-derived ARS was quite efficient at maintaining autonomous elements in *P. pastoris*. Alternatively, the apparent strength of the ARS may be the result of a lack of sequence homology between pYA2 and the *Pichia* genome and, therefore, a consequence of the plasmid not being able to recombine with chromosomal DNA. To distinguish between the two explanations, a 2.7-kb *Bgl*II fragment from the *Pichia* genome (encoding the *Pichia HIS4* gene) was inserted into the *Bam*HI site of pYA2, and the recombinant plasmid, pYJ28, was transformed into GS115. pYJ28 integrated into the *Pichia* genome after about 10 generations in selective medium (Table 1), thus indicating the lack of homology as the reason for the autonomous behavior of pYA2.

Characterization of the *Pichia HIS4* gene. The location of the *Pichia HIS4* gene in the cloned *P. pastoris* genomic fragment of pYA4 was deduced by transforming *S. cerevisiae* 6657-9B and *P. pastoris* GS115 with portions of the fragment. The smallest subfragment which was capable of transforming both yeasts at high frequency was a 2.7-kb *Bgl*II fragment (Fig. 1). The *Pichia* activities equivalent to those encoded by the *Saccharomyces HIS4A* and *HIS4B* loci appeared to lie within the *Bgl*II-*Sal*I fragment nearest the

TABLE 1. Properties of *Pichia* and *Saccharomyces HIS4* gene-containing plasmids in *P. pastoris*

Plasmid	Source of *HIS4* gene	Transformation frequency (colonies/μg)	Generation time[a]	Generations as autonomous element[b]	Copy no.[c]	Stability[d]
pYA4	*P. pastoris*	9.7×10^4	2.7	10	6	71
pYJ8	*P. pastoris*	5.0×10^1	2.3	<10		
pYJ8Δ*Cla*	*P. pastoris*	5.0×10^1	2.3	<10		
pYA2	*S. cerevisiae*	1.6×10^4	3.1	>50	6	90
pYJ28	*P. pastoris* and *S. cerevisiae*	1.2×10^4	3.0	10	ND	ND

[a] Hours per generation in selective medium. The generation time of GS115 in the same medium supplemented with 20 μg of histidine per ml is 2.3 h.

[b] Transformants were grown logarithmically for 50 generations in selective medium, and the state of the plasmid sequences (autonomous versus integrated) was determined by Southern hybridizations of labeled pBR322 to undigested yeast DNAs as described in Materials and Methods.

[c] Transformed cells were grown in selective medium. Plasmid copy numbers are relative to that of the *Pichia* genomic *HIS4* gene and were estimated by Southern blot hybridizations as described in Materials and Methods. ND, Not determined.

[d] Stability numbers are the percentage of cells which lost the His$^+$ phenotype during 10 generations of growth in nonselective medium.

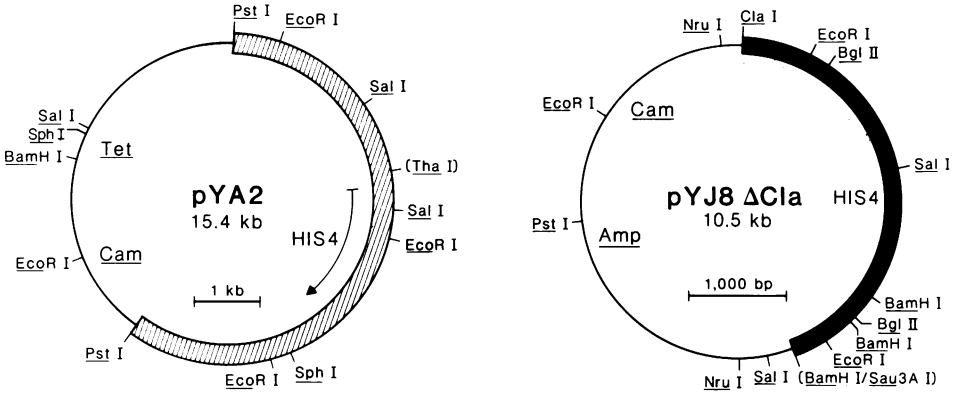


FIG. 3. Restriction enzyme maps of pYA2 and pYJ8Δ*Cla*. Plasmid pYA2 consists of pBR325 (thin line) and a 9.4-kb fragment which contains the *S. cerevisiae HIS4* gene (hashed line). The *P. pastoris* ARS selection plasmid pYJ8Δ*Cla* is composed of pBR325 sequences (thin line) and a 4.5-kb fragment which contains the *Pichia HIS4* gene (dark box). The position and direction of transcription of the *HIS4* gene is shown by the arrow. Tet, Tetracycline; Amp, ampicillin; Cam, chloramphenicol.

*Bam*HI sites since plasmids which contained the 1.5-kb fragment complemented the *his4AB*$^-$ *Saccharomyces* host. The *Pichia HIS4C* equivalent loci appeared to be located within the other *Sal*I-*Bgl*II fragment since this fragment hybridized to a probe which contained the 3′ end of the *Saccharomyces HIS4* gene (including the *HIS4C* region) and transformed GS115. The latter observation also suggests that the mutation in GS115 lies within the *Pichia* genome on the 1.2-kb *Sal*I-*Bgl*II fragment. The DNA sequence of about 1 kb of the 5′-terminal *Bgl*II-*Sal*I fragment was determined, and the probable amino acid sequence encoded by this portion of the *Pichia HIS4* gene was compared with that of the *Saccharomyces HIS4* gene (16). Four regions of significant amino acid sequence homology were found (6 of 12, 9 of 11, 26 of 38, and 12 of 16 amino acids) which were in the same order and about equally spaced in the two sequences.

To examine the *Pichia HIS4* gene for ARS activity, we inserted a 6.4-kb *Eco*RV-*Sph*I fragment from pYA4 containing the entire 6.0 kb of *Pichia* DNA into *Eco*RV- and *Sph*I-digested pBR325. This vector, pYJ8, transformed GS115 at a low frequency (Table 1). Experiments in which labeled pBR325 was hybridized to GS115-pYJ8 transformants indicated that the vector integrated into the *Pichia* genome (data not shown). Thus, neither pBR325 nor the 6.0-kb *Pichia HIS4* fragment contains significant PARS activity. In further analysis of vectors composed of the *Pichia HIS4* gene and portions of YEp13, the 3.9-kb *Saccharomyces LEU2* gene fragment was shown to contain the primary ARS activity of pYA4, and the 2.2-kb *Eco*RI fragment from the 2μm plasmid was found to have low but significant activity in *P. pastoris*.

Isolation and characterization of other PARSs. To maintain autonomously replicating plasmids which contain homologous DNA in *Pichia* cells, it was necessary to obtain stronger PARSs. In *S. cerevisiae* the presence of a sequence with ARS activity increases the transformation frequency of a plasmid 10^3- to 10^5-fold (26, 47). This property has provided a basis for the selection of *S. cerevisiae* ARSs (44). However, from reports on other yeast transformation systems (2, 15) and from preliminary studies on the *Pichia* system (see below), it was evident that DNA fragments isolated on the basis of ARS activity in *S. cerevisiae* may not have ARS activity in *P. pastoris*. Therefore, it was necessary to isolate PARSs by selection in *P. pastoris*.

As the first step in the isolation of PARSs, a library of *Pichia* DNA was constructed in pYJ8Δ*Cla*, a derivative of pYJ8 which contains only a single *Cla*I site (Fig. 3). DNA from GS115 was partially digested with *Taq*I, and 5- to 10-kb fragments were inserted into the *Cla*I site of pYJ8Δ*Cla*. The library transformed GS115 at a frequency 10^3 times greater than pYJ8Δ*Cla* (Table 1). A sublibrary was formed by recovering plasmids from about 10^4 GS115 transformants. This sublibrary transformed GS115 at a frequency 10^4 times greater than pYJ8Δ*Cla*. Forty of the His$^+$ yeast colonies from the sublibrary transformation were picked and streaked onto agar plates containing selective medium. Cells from each of the 40 streaks were grown in selective medium, and plasmids were recovered from the yeast DNAs by transformation of *E. coli*. Four plasmids, pYA63, pYA77, pYA90, and pYA96, which were recovered from the yeast DNA preparations and which produced the most ampicillin-resistant *E. coli* colonies were analyzed further.

Results of analysis of pYA63, pYA77, pYA90, and pYA96 showed that each transformed *P. pastoris* at a very high frequency and contained inserts of 0.2, 2.4, 0.4, and 4.8 kb, respectively. The small size of the inserts was unexpected since the original library was constructed with 5- to 10-kb fragments of *Pichia* DNA. Yet only pYA96 contained an insert in that size range. Since no deletions or rearrangements of the pYJ8Δ*Cla* vector portion of the recombinant plasmids were detected by restriction analysis, it appears that our screening procedure may have selected rare recombinants with small *Pichia* DNA inserts.

To confirm that each of the PARSs was in fact a *P. pastoris* DNA sequence and to determine the type of sequence from which each originated (repeated versus single copy), each of the four PARS plasmids was labeled and hybridized to restriction digests of *Pichia* genomic DNA by the method of Southern (42). The amount of labeled plasmid which hybridized to the *Pichia* fragments relative to that which hybridized to the genomic copy of the *Pichia HIS4* fragment suggested that the PARS fragments in pYA63, pYA90, and pYA96 originated from single-copy-number sequences but that the ARS fragment from pYA77 was from a sequence repeated about 10 times per copy of the genomic *HIS4* gene.

The PARs plasmids were transformed into *S. cerevisiae* to determine whether they could function in *Saccharomyces* species. None appeared to have significant ARS activity in *S. cerevisiae* (data not shown).

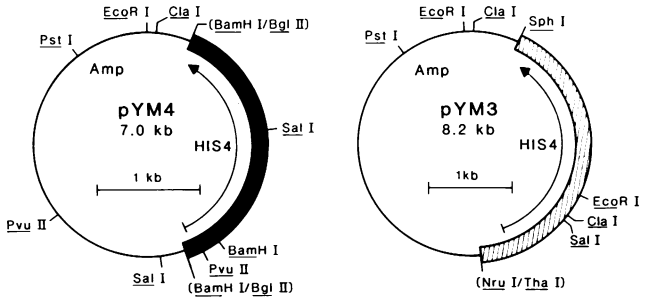


FIG. 4. Restriction enzyme maps of pYM4 and pYM3. The plasmids are composed of pBR322 sequences (thin lines) and either a 2.7-kb fragment which contains the *P. pastoris HIS4* gene (thick line) or a 3.8-kb fragment which contains the *S. cerevisiae HIS4* gene (hashed line). Amp, Ampicillin.

Since the *Pichia* DNA fragments in pYA63, pYA77, pYA90, and pYA96 showed similar ARS characteristics, only the two smallest fragments containing ARSs from pYA63 (PARS1) and pYA90 (PARS2) were further characterized. To determine whether the ARS activity of the two fragments was dependent on plasmid sequences adjacent to the fragments, the *Taq*I fragments which contain the PARSs were inserted into the pBR322 *Cla*I sites of pYM4 and pYM3 (Fig. 4). Plasmid pYM4 is composed of the *Pichia HIS4* gene on a 2.7-kb *Bgl*II fragment inserted into the *Bam*HI site of pBR322. Plasmid pYM3 contains the *Saccharomyces HIS4* gene on a 3.8-kb *Sph*I-*Tha*I fragment inserted into *Sph*I- and *Nru*I-cut pBR322. Note that neither pYM4 nor pYM3 have the 1.7-kb fragment containing the chloramphenicol resistance gene immediately adjacent to one side of their pBR-based *Cla*I sites as does pJY8Δ*Cla*. In addition, about 1.0 kb of *Pichia* DNA from the *Cla*I to the *Bgl*II sites which exists in pYJ8Δ*Cla* is not present in either pYM4 or pYM3. Yet Table 2 indicates that either PARS fragment when inserted into either pYM4 and pYM3 conferred the characteristics associated with strong ARS activity. Derivatives of pYJ30 (pYM4 plus PARS1) and pYJ32 (pYM4 plus PARS2) in which the *Taq*I fragments containing PARS1 and PARS2 were inserted in the opposite orientation showed strong ARS-plasmid characteristics in *P. pastoris* as well. Of particular importance are the results of the long-term growth studies with GS115 transformants containing pYJ30 and pYJ32. Both plasmids remained autonomous after 50 generations in selective medium, whereas pYA4 and pYJ28, which contained *S. cerevisiae* fragments with fortuitous PARS activity, integrated into the *P. pastoris* genome (Table 1).

The nucleotide sequence of PARS1 and PARS2 was determined by the dideoxynucleotide chain termination method of Sanger et al. (39) (Fig. 5). PARS1 is a 164-bp *Taq*I fragment with sites for restriction endonucleases *Nru*I, *Bgl*II, and *Hinc*II. PARS2 is a 385-bp *Taq*I fragment with sites for *Sal*I and *Sma*I. Although neither PARS functioned in *S. cerevisiae*, features in common with *Saccharomyces* ARSs were evident (10). First, both PARSs were rich in adenine plus thymine (A+T) base pair content. PARS1 contained a continuous 112-bp sequence which was 78% A+T, and PARS2 contained a 203-bp sequence which was 80% A+T, both of which are significantly higher than the 55 to 65% A+T chromosomal DNA contents reported for *Pichia* species (45). Second (Fig. 5), both PARSs contained at least one 10-of-11-bp match to the *S. cerevisiae* consensus ARS (25, 43). Third, both PARSs contained a number of short (8- to 10-bp) direct and inverted repeats. One almost perfect set of inverted repeats was found in both PARS1 and PARS2. Both PARS inverted repeat (PAIR) sequences were 9 bp in length (the 3′ repeat of PARS1 inverted repeat contained one additional base pair) and were separated by about 80 bp (Fig. 5).

DISCUSSION

We developed a methylotrophic yeast, *P. pastoris*, as a host system for transformations. The system is based on a mutant strain of *P. pastoris*, GS115, which is defective in histidinol dehydrogenase and plasmid vectors which contain either the *P. pastoris* or *S. cerevisiae HIS4* gene as a selectable marker. For DNA transfer, spheroplasts of GS115 were generated and mixed with DNA in the presence of $CaCl_2$ and polyethylene glycol, and transformants were selected by histidine prototrophy.

GS115 was identified as histidinol dehydrogenase defective by screening a collection of histidine auxotrophs for ATP phosphoribosyl-transferase, imidazoleglycerol-phosphate-dehydratase, histidinol-phosphate phosphatase, and histidinol dehydrogenase activities. We elected to screen the *Pichia* His^- collection since histidine requirers were the most frequent auxotroph isolated (George Sperl, unpublished results) and since of the seven known *S. cerevisiae* histidine biosynthetic genes (24), three (*HIS1*, *HIS3*, and *HIS4*) were previously isolated and characterized (16, 20, 21, 46).

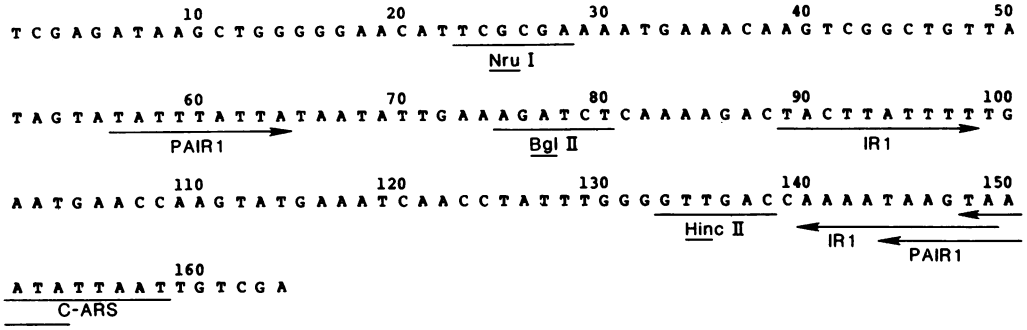
In *S. cerevisiae*, histidinol dehydrogenase is a multifunctional polypeptide which possesses three different enzymatic specificities needed for the biosynthesis of histidine. It is encoded in a single gene, *HIS4* (24). Phosphoribosyl-ATP cyclohydrase, which is required in the third step in the pathway, is the amino-terminal activity of the polypeptide. Phosphoribosyl-ATP pyrophosphohydratase and histidinol dehydrogenase, which are required

TABLE 2. Properties of *Pichia* plasmids which contain PARS1 or PARS2

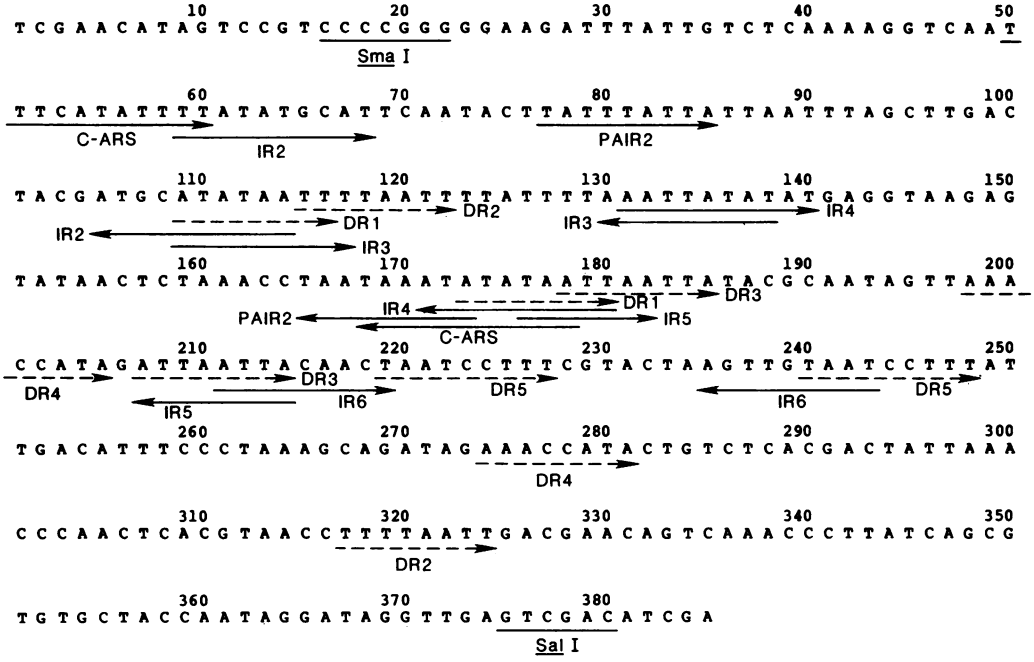
Plasmid	ARS source	Transformation frequency (colonies/μg)	Generation time[a]	Generations as autonomous element[b]	Copy no.[c]	Stability[d]
pYM4		1.1×10^1	2.5			
pYJ30	PARS1	1.8×10^5	2.7	>50	13	50
pYJ32	PARS2	1.7×10^5	2.9	>50	13	51
pYM3		7.0×10^3	4.4	>50	3	97
pYM8	PARS1	1.9×10^5	2.8	>50	ND	48
pYM9	PARS2	1.6×10^5	2.9	>50	ND	59

[a,b,c,d] As described in Table 1 footnotes.

A.



B.



C.

ARS	POSITION	C-ARS
PARS1	147-158	A TTAAT A TTT A
PARS2	49-60	T TTCAT A TTT T
PARS2	167-178	T TATAT A TTT A
CONSENSUS ARS		(A/T)TTTAT(A/G)TTT(A/T)

PAIR1 TATTTATTA . . . N_{81} . . . TAA G TAAATA

PAIR2 TATTTATTA . . . N_{80} . . . TAA TAAATA

for the 2nd and 10th steps in the pathway, are the central and carboxy-terminal activities, respectively. The loci which encode the three active-site domains in the *HIS4* gene are designated *HIS4A*, *HIS4B*, and *HIS4C*, respectively.

As the selectable marker gene for GS115 transformations, the *Pichia* gene which encodes the same activities as that of the *Saccharomyces HIS4* gene was sought. The gene was isolated from a *Pichia* DNA library by its ability to complement a *his4ABC*$^-$ strain of *S. cerevisiae*, and its identity was confirmed by hybridization to the *Saccharomyces HIS4* gene. A 2.7-kb *Bgl*II fragment was eventually identified which could complement both the *his4ABC*$^-$ *S. cerevisiae* and *his4C*$^-$ *P. pastoris* hosts. Additional analysis suggested that the *Pichia* gene is organized in a manner similar to the *Saccharomyces HIS4* gene. Thus, we refer to the *P. pastoris* gene as the *Pichia HIS4* gene and GS115 as *his4C*$^-$. We do not know whether GS115 is *his4A*$^-$ or *his4B*$^-$ or both.

The ability to isolate *Pichia* genes by complementation of the appropriate *Saccharomyces* mutant hosts will be extremely useful in studies of *P. pastoris*, since *P. pastoris* mutants are difficult to generate and since hundreds of *S. cerevisiae* mutants have geen isolated (32) and characterized (7). To date, the *Pichia* genes equivalent to the *Saccharomyces HIS3* and *ARG4* genes have been isolated (F. Gaertner and J. Cregg, unpublished data), and prototrophic colonies have appeared when the *Pichia* DNA-YEp13 library was transformed into *Saccharomyces his1*$^-$ and *ade2*$^-$ mutant hosts (J. Cregg and A. Hessler, unpublished data). It may be possible to use *Pichia* genes isolated in this manner to create desired mutant strains of *P. pastoris* by manipulating *Pichia* gene fragments to form site-specific insertion vectors (37). Such a mutant-generating method might succeed in producing mutants which would be difficult if not impossible to produce by other mutagenesis and selection procedures in *P. pastoris*.

Transformation of *P. pastoris* by the spheroplast generation procedure is very efficient. Preparations of Zymolyase-treated *Pichia* cells routinely contained greater than 99% spheroplasts (i.e., the proportion of total regeneratable protoplasts relative to that of remaining whole cells). Transformation frequencies with some plasmids (Table 2) were consistently about $10^5/\mu g$ or 0.1 to 1.0% of the total regeneratable spheroplast population. The reversion frequency of GS115 at less than 10^{-8} was so low that revertants were rarely seen in transformations and rare events, such as integrative plasmid transformations, were easily detected.

A number of *S. cerevisiae* DNA fragments were examined for ARS activity in *P. pastoris* by recombining the *Saccharomyces* fragments onto a plasmid which contained the *Pichia HIS4* gene. No significant correlation in ARS activity between the two yeasts was observed. A plasmid which contained the *Saccharomyces* ARS1 on a 1.45-kb *Eco*RI fragment (47, 51) transformed *P. pastoris* at low frequency and integrated into the *Pichia HIS4* locus. Plasmids which contain the 2μm plasmid ARS on a 2.2-kb *Eco*RI fragment (8) transformed *P. pastoris* at a high frequency but integrated into the *Pichia HIS4* locus soon after transformation. A plasmid containing the entire 2μm plasmid showed no better autonomous behavior. On the other hand, 2μm fragments which have little or no ARS activity in *S. cerevisiae*, a 3.9-kb *Pst*I fragment containing the *LEU2* gene (22) and a 9.4-kb *Pst*I fragment with the *Saccharomyces HIS4* gene (21), did have substantial ARS activity in *P. pastoris*. In other yeasts, as well, there appears to be little correlation in the ability of a fragment which was selected on the basis of ARS activity in *S. cerevisiae* to support autonomous replication in a heterologous host. Neither *Saccharomyces* ARS1 nor the 2μm plasmid ARS functions in *K. lactis* or *K. fragilis*, and a vector which contained the entire 2μm plasmid transformed only at low frequency (14, 15). In *S. pombe* some (but not all) plasmids which contain the entire 2μm plasmid transform at high frequency and are autonomous (2). However, a set of eight plasmids each of which contained a different *S. cerevisiae* ARS fragment transformed *S. pombe* at low frequencies.

Since the *S. cerevisiae* fragments we examined did not support the autonomous replication of plasmids in *P. pastoris* particularly well, DNA fragments with stronger ARS activity were isolated by a modified version of the method used to isolate ARSs in *S. cerevisiae* (44; see references 31 and 48 for reference lists). The method has been successfully performed in *K. lactis* to isolate *K. lactis* ARS fragments as well (14). For the isolation of *P. pastoris* ARSs, selective pressure to enrich for "strong" ARSs was applied by passing the *P. pastoris* ARS-selection library through *P. pastoris* cells twice and by growing the resultant *Pichia* transformants in selective medium before the final plasmid recovery step.

Two of the PARSs, PARS1 and PARS2, were extensively characterized. Experiments in which the PARSs were subcloned into other *Pichia* plasmids demonstrated that the small ARS fragments are not dependent on external sequences for activity. The DNA sequence results showed that PARS1 and PARS2 share features with each other and with *S. cerevisiae* ARSs such as a high A+T content and homology to the *S. cerevisiae* consensus ARS (9).

Given these similarities it was surprising that plasmids which contained PARS fragments transformed *S. cerevisiae* at such low frequencies (0 to 50/μg). However, Broach et al. (10) noted that other sequences, such as those from *MAT***a** and *MAT*α, have high A+T content and a close match to the consensus ARS and yet do not promote autonomous replication. Thus, it appears either that sequences within such ARS-like fragments inhibit ARS function or that these common features are not sufficient for ARS activity. The fact that ARS fragments which function in *P. pastoris* frequently did not in *S. cerevisiae* and vice versa suggests that although some sequence features are common between the two yeasts, other sequences required for ARS activity are host species specific.

Most aspects of plasmid behavior in *P. pastoris* are similar to those reported for plasmids in *S. cerevisiae*. First, plasmids which transform at low frequency integrate into the genome of the host. The most common result of integrative

FIG. 5. (A) DNA sequence of the 164-bp *Taq*I fragment which contains PARS1. In plasmids pYA63, pYJ30, and pYM8 (see Table 2), the *Nru*I site of PARS1 is oriented closest to the *HIS4* gene containing portions of the plasmids. (B) DNA sequence of the 385-bp *Taq*I fragment which contains PARS2. In plasmids pYA90, pYJ32, and pYM9, the *Sma*I site of PARS2 is oriented closest to the *HIS4* gene-containing portions of the plasmids. The position and direction of sequences with homology to the *S. cerevisiae* consensus ARS (C-ARS), of the PARS inverted repeat (PAIR) sequences, and of other direct repeat (DR) and inverted repeat (IR) sequences are denoted by the arrows. (C) DNA sequences of interest from PARS1 and PARS2. *P. pastoris* nucleotide positions from the PARSs which match the *S. cerevisiae* consensus ARS are denoted with asterisks. PAIR1 and PAIR2 are the PARS inverted repeat sequences from PARS1 and PARS2, respectively.

TABLE 3. Comparison of *P. pastoris* and *S. cerevisiae* strains transformed with ARS-based plasmids

Plasmid	Host	ARS source	Generation time[a]	Stability[b]	Reference
pYM8	*P. pastoris*	PARS1	2.8	48	This study
pYM9	*P. pastoris*	PARS2	2.9	59	This study
YRp12	*S. cerevisiae*	ARS1	4.0	95	Stinchcomb et al. (44)
Y-m8	*S. cerevisiae*	Mouse	4.6	72	Roth et al. (36)

[a] Hours per generation in a selective medium. The generation times reported for strains of both yeasts in nonselective medium were between 2.0 and 2.5 h.
[b] As described in Table 1, footnote *d*.

transformation events with circular plasmid molecules in *P. pastoris* is the same "addition" type observed in *S. cerevisiae*, that is, a direct duplication of the homologous yeast sequences flanking the foreign vector sequences (22). Second, as in *S. cerevisiae*, plasmids which transform *P. pastoris* at high frequencies are autonomous for at least a short period after transformation. Third, both *Pichia* and *Saccharomyces* ARS-based vectors are lost from cells growing in nonselective media. And fourth, most plasmids recovered from *Pichia* cells via transformation of the yeast DNAs into *E. coli* appear from restriction analysis to be unchanged relative to the same plasmids before transformation.

Some aspects of ARS-based plasmid behavior in *P. pastoris* are different from those in *S. cerevisiae*. First, *Pichia* plasmids which contained sequences homologous to the *Pichia* genome appeared to integrate at high frequency, whereas the integration of an ARS-containing plasmid in *S. cerevisiae* is a relatively rare event. Second (Table 3), *Pichia* strains which contained autonomous plasmids generally grew faster in selective medium than *Saccharomyces* strains with ARS-based plasmids (36, 44). And third, the loss rate of ARS-based plasmids from *Pichia* cells growing in nonselective medium was generally lower than rates reported for plasmids in *S. cerevisiae*. All of these observations could be explained as the predicted result of a higher general homologous recombination frequency in *P. pastoris* relative to *S. cerevisiae*. Plasmids with homology to the *Pichia* genome would recombine and integrate more frequently. Therefore, an ARS which maintained a plasmid as an autonomous element in *P. pastoris* must be relatively strong, a characteristic which would be reflected in a fast growth rate for transformed yeast strains in selective medium and in a low rate of loss from cells growing in nonselective medium.

A major factor which appeared to determine the state of a plasmid in *Pichia* cells (autonomous versus integrated) is whether sequences homologous to the *Pichia* genome were present on the plasmid. The stable autonomous state of plasmids which lack host sequence homology has obvious practical value for the *Pichia* transformation system. Since the *Saccharomyces HIS4* gene functions in *P. pastoris* and yet has little sequence homology to the *Pichia HIS4* gene equivalent, the use of the *Saccharomyces HIS4* gene (or other heterologous selective marker genes) should increase the length of time plasmids remain autonomous in *Pichia* cells.

Finally, for the first time a transformation system is available for studies with methylotrophic yeasts. At least two subjects are of immediate interest. The first is the regulation of expression of genes by methanol. Preliminary results with plasmids which contain sequences from the *P. pastoris* AO gene fused to those of the *E. coli lacZ* gene indicate that beta-galactosidase expression is regulated in *Pichia* cells transformed with these plasmids in the manner consistent with previous studies on the regulation of AO synthesis (Juerg Tschopp, personal communication). The second subject is the method by which enzymes such as AO and catalase are compartmentalized into peroxisomes. Protein and DNA sequence results of AO and its gene indicate that AO has no cleaved amino-terminal signal sequence (17). Using the *Pichia* transformation system, it may now be possible to determine sequences involved in peroxisomal packaging by constructing a series of gene fusion vectors and examining the behavior of their gene products in *P. pastoris*.

ACKNOWLEDGMENTS

We thank G. Sperl for the *P. pastoris* auxotrophic mutant strains and G. Fink for the gift of *S. cerevisiae* strains and the *HIS4* gene-containing plasmid. We thank T. Gingeras, M. Harpold, G. Thill, J. Tschopp, and B. Smiley for their assistance and discussion. In addition, we wish to thank D. Stroman of Phillips Petroleum Co. and T. Hunter and G. Wahl of The Salk Institute for their discussions and comments throughout the project. We thank Susan Konchal and Pat Thomas for help in preparing this manuscript.

This research was supported by Phillips Petroleum Co.

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