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MOLECULAR CHARACTERIZATION OF A SACCHAROMYCES PLASMID CONTAINING THE *HIS4* GENE

by

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In a previous study, genetic transformation in yeast was carried out with a mixture of the yeast plasmid 2-micron DNA and total yeast DNA, which was treated with restriction endonuclease PstI and DNA ligase. Strains were derived that contain a plasmid which carries the *HIS4* gene. In the present study new plasmids have been constructed by combining the *HIS4* carrying yeast plasmid, as well as parts of it, with bacterial plasmids. The hybrid plasmids were propagated in bacteria and analysed. From the data obtained the original plasmid was inferred to be a 2-micron DNA circle with a 9.4 kb insertion carrying the *HIS4* gene. This structure was confirmed by restriction endonuclease analysis of plasmid DNA from the original transformant, using molecular hybridization to detect fragments which contain sequences of the 9.4 kb insert. The insert, which is bordered by two PstI sites, was mapped with restriction endonucleases EcoRI, HindIII, and SalI. No BamHI site is present in the insert.

Abbreviations: BSA = bovine serum albumin, cc = closed circles, ccDNA = closed circular DNA, kb = kilobases, oc = open circles.

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1. INTRODUCTION

Genetic transformation in the yeast Saccharomyces cerevisiae is usually carried out with chimaeric plasmid DNA consisting of specific yeast DNA sequences and a bacterial plasmid that allows the convenient selection and propagation of the chimaeric plasmid in bacteria (1, 8, 10, 24). However, we have demonstrated that it is possible to transform yeast with DNA derived solely from yeast without involving foreign DNA (16). In those experiments a mixture of total yeast DNA and the yeast plasmid 2-micron DNA was treated with restriction endonuclease PstI. After ligation, the DNA was used to transform a *his4* deletion mutant to histidine prototrophy. Genetic studies showed that in the transformant C78-H26 the *HIS4* gene is inherited in a non-mendelian way, consistent with the assumption that it is located on an insertion in 2-micron DNA.

The amount of the assumed combined plasmid was too low to be detected in stained gel electropherograms of plasmid DNA from the transformant. For the molecular characterization of the *HIS4* carrying yeast plasmid we therefore decided to clone the plasmid and/or the *HIS4* containing fragment on bacterial plasmids for propagation in E. coli.

Since the *HIS4* gene product may not complement any known E. coli mutation, we selected the *HIS4* carrying plasmids by transformation of a *his4* yeast strain after propagation in E. coli.

In this paper we report the insertion in bacterial plasmids of both the 2-micron-*HIS4* pure yeast plasmid and of the *HIS4* carrying region. Furthermore, restriction endonuclease maps of these DNA molecules are presented together with a molecular characterization of the *HIS4* carrying pure yeast plasmid in the original transformant C78-H26.

2. MATERIALS AND METHODS

2.1. Strains and plasmids

Yeast strains used in this study are given in Table I. The E. coli K-12 strain used in transformation was JF1161 (r_k^- m_k^+ leu gal met) obtained from N. FIIL, Institute of Microbiology, University of Copenhagen. Plasmids pBR325 (2) and pJC75-58 (4) were obtained from F. BOLIVAR and J. COLLINS, respectively.

2.2. Media and growth

The media used for growth of yeast have been described (16). E. coli was grown in LB (1% Difco Bacto tryptone, 0.5% Difco Bacto yeast extract, 1% NaCl, 0.1% glucose). Plates were solidified with 2% Difco Bacto agar. Ampicillin and tetracycline (Sigma) were used at concentrations of 50 and 10 $\mu g \cdot ml^{-1}$, respectively. Growth of E. coli was at 37 °C, except when the strain contained a plasmid derived from pJC75-58, in which case 30 °C was used. Liquid cultures were grown with shaking.

Table I.

Yeast strains used in this study.

Strain	Genotype	Source
C75-M26	α *his4-29 leu1-1*	KIELLAND-BRANDT et al. (16)
C78-H26	α *his4-29*[HIS4]*leu1-1*	–
C78-H23/1	α *his4-24*[HIS4]*ade2-1*	–
CB-2g-29	α *his4-29 trp1 ilv1*	T. NILSSON-TILLGREN
C76-S1-27C	a *his4-24 his1 ade1*	S. HOLMBERG
C79-D765	a/α *his4-24/his4-29 his1/HIS1 ade1/ADE1 TRP1/trp1 ILV1/ilv1*	Diploid of CB-2g-29 × C76-S1-27C
C79-H845	a/α *his4-24/his4-29*[HIS4]*his1/HIS1 ade1/ADE1 TRP1/trp1 ILV1/ilv1*	This study
C79-H846	–	–
C79-H847	–	–
C79-H848	–	–

2.3. Plasmid DNA

Plasmid DNA from yeast was prepared according to LIVINGSTON and KLEIN (18) except that ethidium ions were removed with a column of Dowex 50 (1 ml bed).

For plasmid DNA preparation from E. coli, a cleared lysate was prepared according to KATZ et al. (15) from cells that had been killed with 0.1% diethyl pyrocarbonate (25). Per ml of lysate were added 0.95 g CsCl and 0.1 ml 1% ethidium bromide. Centrifugation was carried out in a Beckman 50 Ti rotor at 38,000 rpm and 15 °C for 48 hours. The plasmid band was collected, freed from ethidium ions by passage through Dowex 50 and precipitated with 3 volumes of 70% ethanol at 4 °C overnight. When plasmids derived from pJC75-58 were isolated, the copy number was amplified by the addition of chloramphenicol (150 $\mu g \cdot ml^{-1}$) to the logarithmic phase culture followed by shaking for 16 hours at 30 °C.

For rapid screening of plasmids from bacterial transformants, the following procedure was used: 50 ml cultures were grown overnight in LB, killed with 0.1% diethyl pyrocarbonate (25) and a cleared lysate (15) was prepared. The lysate was heated to 65 °C for 15 minutes and the precipitate was removed by centrifugation at 5,000 rpm for 5 minutes in the Sorvall SS34 rotor. The DNA was precipitated from the supernatant by the addition of NaCl and polyethylene glycol to final concentrations of 0.5 M and 10% (w/v), respectively. After standing at 0 °C for 1 hour, the precipitate was collected by centrifugation at 10,000 rpm for 15 minutes in the SS34 rotor. The pellet was dissolved in 0.4 ml of 10 mM-Tris-HCl, 0.1 mM-EDTA, pH 7.5 and a sample taken for electrophoresis. For restriction enzyme treatment the solution was made 0.5 M in sodium acetate and the DNA precipitated with 3 volumes of cold absolute ethanol. After standing at −80 °C for 30 minutes the precipitate was spun down at 13,000 rpm in a Beckman Microfuge B for 4 minutes, washed twice in absolute ethanol and dissolved in the appropriate buffer.

2.4. Restriction and ligation reactions

Cleavage with restriction endonuclease EcoRI (Boehringer) was carried out in 100 mM-Tris-HCl (pH 7.5), 50 mM-NaCl, 5 mM-$MgCl_2$, 100 $\mu g \cdot ml^{-1}$ bovine serum albumin (BSA), 1 mM-dithiothreitol; PstI and BamHI (Boehringer or New England Biolabs) in 50 mM-NaCl, 6 mM-Tris-HCl (pH 7.4), 6 mM-$MgCl_2$, 6 mM-2-mercaptoethanol, 100 $\mu g \cdot ml^{-1}$ BSA; HindIII and SalI (New England Biolabs) in 60 mM-NaCl, 10 mM-Tris-HCl (pH 7.4), 7 mM-$MgCl_2$, 6 mM-2-mercaptoethanol, 100 $\mu g \cdot ml^{-1}$ BSA. When DNA was to be digested with two restriction endonucleases requiring different ionic conditions, it was precipitated with ethanol and redissolved in the appropriate buffer before the second treatment. Standard conditions were to use 1 unit of enzyme per µg of DNA for 2 hours at 37 °C, but sometimes it was found necessary to increase the amount of enzyme to get proper digestion. After the reaction the samples were always heated to 70 °C for 5 minutes in order to inactivate the nuclease. Ligation with T4 DNA ligase (New England Biolabs) was at 14 °C in 10 mM-Tris-HCl (pH 7.9), 10 mM-$MgCl_2$, 100 $\mu g \cdot ml^{-1}$ BSA, 0.1 mM-ATP, 10 mM-dithiothreitol.

Alkaline phosphatase from Boehringer (No. 108 148) was further purified by gel filtration (7). Removal of 5'-phosphate from the ends of DNA was carried out with 0.4 units of this enzyme per µg of DNA in 0.1 ml of 10 mM-Tris-HCl, pH 8.0 for 30 minutes at 37 °C and stopped by two extractions with phenol, two subsequent ether extractions and precipitation with ethanol.

2.5. Transformation

The procedure for transformation of yeast has been described (1, 16). The amount of DNA and number of protoplasts used are given in the text for each experiment.

For transformation of E. coli the calcium chloride method of MANDEL and HIGA (19) was used with the following modifications: The DNA was added as a solution in 10 mM-Tris-HCl (pH 7.5), 10 mM-$CaCl_2$, 10 mM-$MgCl_2$ and the heating step was at 40 °C for 5 minutes. Before plating or inoculation to media containing tetracycline or ampicillin, 0.5 ml LB medium was added and the cell suspension incubated for 30 minutes at 30 °C to allow expression of the antibiotic resistance gene.

2.6. In vitro packaging of DNA and transduction

In vitro packaging of ligated DNA into bacteriophage particles and subsequent transduction of E. coli were performed according to COLLINS and HOHN (5). The biological activity of endogenous DNA in the packaging cell extract was destroyed by UV irradiation (12).

2.7. Gel electrophoresis of DNA

DNA was separated according to size by electrophoresis in agarose as previously described (16), except that a lower voltage (1.5 $V \cdot cm^{-1}$) and a longer time (18 hours) were used. A gel concentration of 0.7% was used unless otherwise stated. Bacteriophage λ restriction fragments were used as size markers (20): Cleaved with EcoRI, 21.7, 7.52, 5.83, 5.64, 4.85 and 3.48 kb; cleaved with EcoRI and HindIII, 21.7, 5.15, 5.00, 4.27, 3.48, 1.98, 1.90, 1.59, 1.37, 0.94, 0.83 and 0.56 kb; not cleaved, 49.0 kb. Electropherograms are presented with the origin at the upper edge.

2.8. Detection of specific DNA sequences in electropherograms by molecular hybridization

Electrophoretically separated DNA fragments were transferred from the 6 mm thick gel slabs to sheets of cellulose nitrate filter (Millipore, HAWP 000 10) according to SOUTHERN (23) with the following modifications: Before transfer, the gel slab was placed in 0.1 M-sodium citrate, pH 2.7 for 3 hours, 0.01 M of the same buffer for 1 hour, 0.2 M-NaOH, 0.6 M-NaCl for 2 hours and finally in 1 M-Tris-HCl (pH 7.2), 1.5 M-NaCl for 3 hours. To accomplish the transfer, 1.5 M-NaCl, 0.15 M-sodium citrate (10 × SSC) was passed through the gel (23) overnight. The filter sheet was dried at room temperature, baked for 2 hours at 80 °C and rolled into a glass tube in which all subsequent incubations of the filter were carried out (hybridization, washings etc.). Hybridization of labelled DNA to the filter-bound DNA was performed as described by DENHARDT (6) with the modifications of JEFFREYS and FLAVELL (14) using $1.5 \cdot 10^7$ cpm of ^{32}P-labelled and denatured pC502 DNA for 2 days. The labelled plasmid DNA ($10^7 - 10^8$ cpm · μg^{-1}) was prepared by nick translation (22) using [α-^{32}P]-dTTP (The Radiochemical Centre, Amersham, 400 $Ci \cdot mmol^{-1}$). Bovine serum albumin, polyvinylpyrrolidone (PVP-360), salmon DNA (type III), polyadenylic acid (type I), dATP, dGTP, dCTP and deoxyribonuclease I (type DN-25) were purchased from Sigma, Ficoll 400 was from Pharmacia and E. coli DNA polymerase I was kindly provided by H. KLENOW, Biochemical Institute B, University of Copenhagen. After hybridization and washings the filter was dried at room temperature and autoradiographed. The autoradiogram presented in this paper was made by a 40 hour exposure of a Kodak X-Omat RP film at −80 °C.

2.9. Containment

Experiments involving recombinant DNA were registered with the committee on genetic engineering of the Danish National Research Councils. Yeast cells containing DNA sequences from pBR325 or pJC75-58 were handled under P1 conditions (9) and E. coli cells and bacteriophage particles containing yeast DNA sequences were handled under P2 conditions (9).

3. RESULTS

3.1. Transforming activity of closed circular DNA from C78-H26 [HIS4]

Crude nucleic acid from the strain C78-H26 can retransform *his4* strains to histidine prototrophy (16). Assuming that the *HIS4* gene in C78-H26 is carried on closed circular DNA (ccDNA), we wished to determine the transforming activity of this DNA. Chromosomal DNA was partly removed from a lysate of the strain (11, 18) and the remaining DNA was subjected to density separation in cesium chloride and ethidium bromide (18). The separate bands of ccDNA and relaxed DNA were collected, freed from ethidium with Dowex 50 and precipitated with 3 volumes of 70% ethanol. Strain C79-D756 *(his4-24/his4-29)* was transformed with both DNA samples. The transformation frequencies are given in Table II. ccDNA shows the highest transforming activity. The activity of the relaxed DNA is assumed to be due to nicked circles of the transforming plasmid. The transforming activity is 100 fold lower than BEGGS (1)

Table II.

Transformation frequencies of ccDNA and relaxed DNA isolated from strain C78-H26 and strain C75-M26. 10^9 protoplasts of strain C79-D756 were used for transformation.

DNA	DNA concentration ($\mu g \cdot ml^{-1}$)	No. *HIS4* colonies $\cdot \mu g^{-1}$
ccDNA from C78-H26 [HIS4]	4.9	300
relaxed DNA from C78-H26 [HIS4]	490	36
ccDNA from C75-M26 *(his4-29)*	4.9	0
relaxed DNA from C75-M26 *(his4-29)*	490	0

found for a 2-micron DNA-pMB9-*LEU2* plasmid, consistent with the notion that only a small fraction of the plasmid DNA in C78-H26 is a 2-micron DNA-*HIS4* plasmid. DNA from the *his4* parent strain C75-M26 did not have any transforming activity (Table II).

3.2. Combination of the *HIS4* carrying yeast plasmid with cosmid pJC75-58

The first step in the characterization of the *HIS4* carrying plasmid in C78-H26 was to combine it with a bacterial plasmid for propagation in E. coli. Since the *HIS4* carrying plasmid comprises only a minority of the ccDNA of C78-H26, an efficient method was required. Cosmids, bacterial plasmids which are packageable in vitro into bacteriophage particles, provide such a method (5). This method has the additional feature of preferentially incorporating large pieces of DNA, which was an advantage as we expected the *HIS4* carrying plasmid to be larger than the 2-micron DNA present in excess.

The cosmid pJC75-58 carries a gene for resistance to ampicillin which allows selection of bacterial transductants; selection of the hybrid cosmids containing the *HIS4* region was achieved by subsequent transformation of yeast cells with ccDNA from the ampicillin resistant population. The hybrid plasmids from individual yeast transformants were finally propagated in E. coli. A flow diagram of the cloning procedure is outlined in Figure 1.

The cosmid contains two cleavage sites for PstI (4), and as the *HIS4* carrying plasmid is also expected to carry two such sites (16), both plasmid preparations were treated with PstI to partial cleavage. Two μg of ccDNA from C78-H26 and 20 μg of cosmid pJC75-58 DNA were treated in this way. Electrophoretic analysis indicated that about 5% of the 2-micron DNA, and about 60% of the cosmid, had been cut once per molecule. We assume that also a reasonable amount of the *HIS4* carrying plasmid in the yeast ccDNA was cut once per molecule. The digests were combined and treated with 1 unit of T4 DNA ligase at a DNA concentration of 300 $\mu g \cdot ml^{-1}$ for 2 days. Electrophoretic analysis indicated that most DNA had been ligated to long linear pieces. Eleven μg of the ligated DNA were subjected to in vitro packaging into

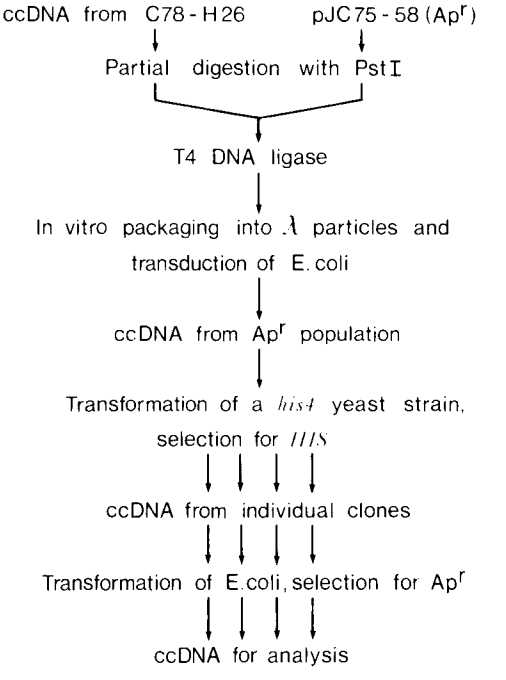


Figure 1. Strategy employed for combination of the *HIS4* carrying yeast plasmid with cosmid pJC75-58.

bacteriophage λ particles (5) which were subsequently used for transduction of E. coli. Plating of a small sample showed that about 10^4 cells in total had been transduced to ampicillin resistance. Expecting that some of the cells had been transduced with a 2-micron DNA-*HIS4*-pJC75-58 plasmid we grew the rest of the cells in liquid culture with ampicillin, whereafter ccDNA was extracted and purified. When 10^9 protoplasts of yeast strain C79-D756 *(his4-24/his4-29)* were subjected to transformation with 32 μg of this DNA, 26 histidine prototrophs were obtained. Four transformants, designated C79-H845 through C79-H848, were chosen at random. The transformants showed instability for histidine prototrophy like C78-H26, although variations between the four strains were observed. Histidine prototrophy was inherited in a non-mendelian way when the strains were sporulated and the tetrads analysed as reported for C78-H26 (16).

The four strains were propagated in minimal medium and ccDNA was extracted and purified. E. coli was transformed with the four DNA samples, giving between 0.3 and 150 ampicillin resistant colonies per μg of DNA. In each case one resistant bacterial clone was grown in liquid culture and ccDNA was extracted and purified.

3.3. Analysis of *HIS4* carrying cosmids

The four DNA preparations were analysed for transforming ability (Table III) and by electrophoresis of restriction endonuclease digests (Figure 2). It appears that the *HIS4* containing plasmids in yeast strains C79-H847 and C79-H848 are not stable in the E. coli rec$^+$ strain which we have used. We assume that this is due to direct repeats of pJC75-58, or part of it, within the chimaeric plasmids which by intramolecular recombination will lead to pure pJC75-58 molecules. If the latter have an advantage in replication over the chimaeric molecules, the result may be partial (Figure 2, lanes g, q and z) or total (lanes h, r and aa) loss of the chimaeric plasmid (cf. also Table III). The *HIS4* carrying plasmids in C79-H845 and C79-H846 retain *HIS4* transforming ability and show no sign of instability during propagation in bacteria. These propagated plasmids are called pC500 and pC501, respectively. They are cleaved with PstI into four fragments (Figure 2, lanes m-q and v-y), three of which by their sizes are suggested to be the two fragments of pJC75-58 (2.05 and 9.9 kb, lanes s and bb) and 2-micron DNA (6.3 kb, lanes t and cc). The fourth fragment of size 9.4 kb is concluded to be the yeast chromosomal fragment containing the *HIS4* gene.

The EcoRI fragments of 2-micron DNA from C78-H26 and plasmids pC500 and pC501 give rise to the following comments. The 2-micron DNA seems identical to that studied by LIVINGSTON and KLEIN (18) and the one called ScpI by CAMERON et al. (3). It is a circle containing a non-tandem inverted repeat and an EcoRI site in each of the two unique regions. Frequent intramolecular recombination in yeast at the inverted

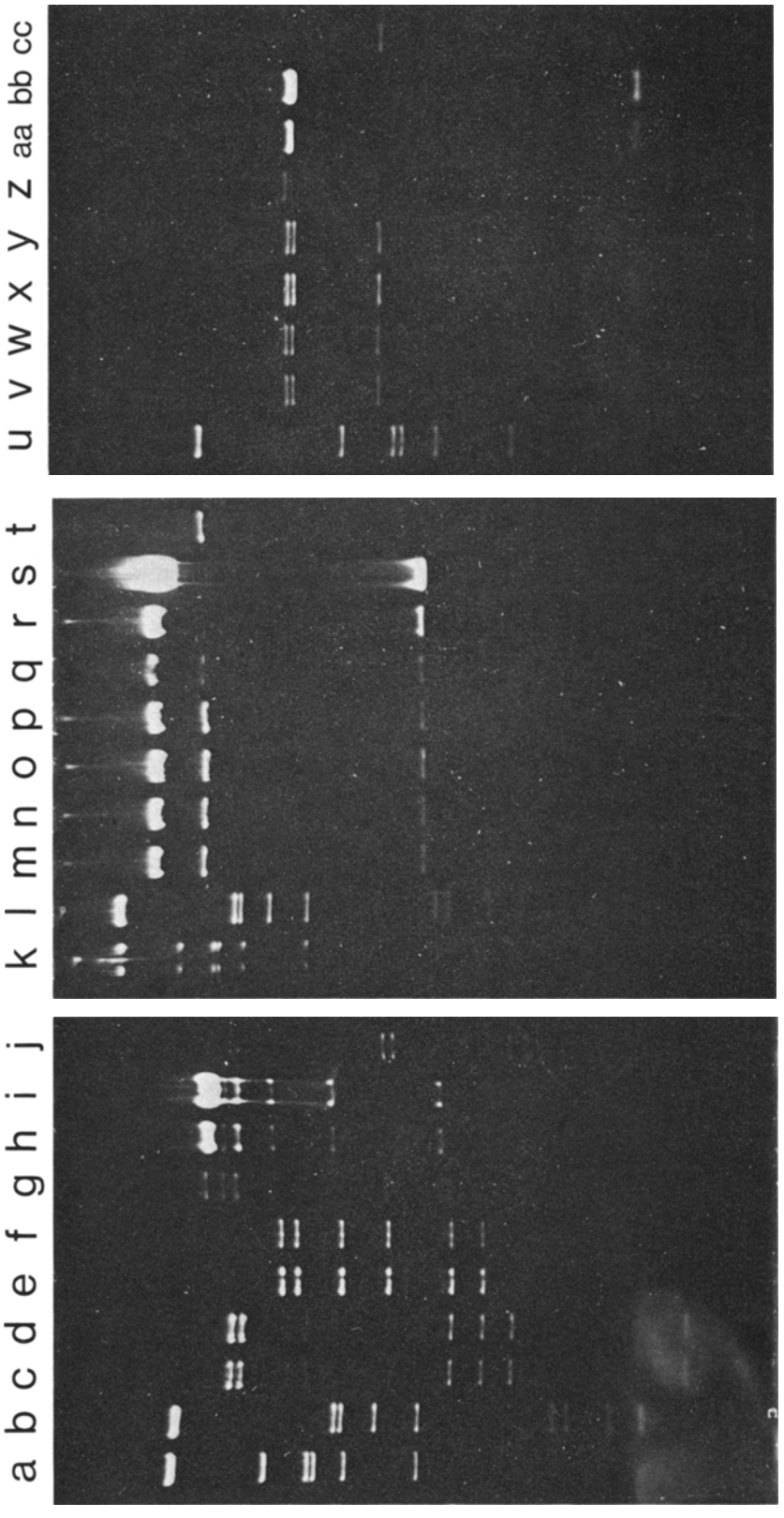
Table III.

Transformation of a *his4* yeast strain with four *HIS4* carrying cosmids propagated in E. coli. 10^9 protoplasts of C79-D756 *(his4-24/his4-29)* were treated with 3–10 μg DNA.

Plasmid originating from yeast strain	Transforming ability (colonies · μg^{-1})
C79-H845	2800
C79-H846	800
C79-H847	300
C79-H848	0

Figure 2. Restriction analysis of *HIS4* carrying cosmids.

Three agarose gel electropherograms of EcoRI (lanes c–j) and PstI (lanes m–t and v–cc) digests of hybrid plasmids propagated in E. coli as well as digests of pJC75-58 (lanes i, s and bb) and 2-micron DNA from C78-H26 (lanes j, t and cc). The plasmids originated from the following yeast strains: Lanes c, d, m, n, v and w are from C79-H845 (plasmid pC500), lanes e, f, o, p, x and y from C79-H846 (plasmid pC501), lanes g, q and z from C79-H847 and lanes h, r and aa from C79-H848. Lanes a, b, k, l and u show molecular weight markers (cf. Materials and Methods). Only the stronger bands in lanes c through i should be considered, since the fragments occurring in less than equimolar amounts must be due to the so-called EcoRI* activity found in EcoRI under certain conditions (21). The gel to the right was lower (0.5 %) in agarose than the two others (0.7 %).


u v w x y z aa bb cc
k l m n o p q r s t
a b c d e f g h i j

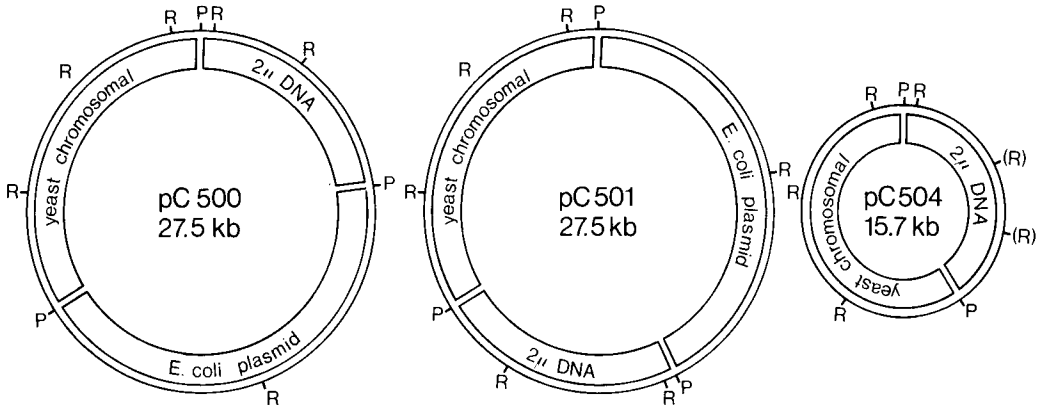


Figure 3. Restriction endonuclease cleavage maps of *HIS4* carrying cosmids (pC500, pC501) and the *HIS4* carrying yeast plasmid of strain C78-H26 (pC504).

The maps of pC500 and pC501 are derived from the data in Figure 2. The map of pC504 is inferred from the maps of pC500 and pC501 and is confirmed by a direct analysis presented in Figure 7. P = cleavage site for PstI; R = cleavage site for EcoRI; (R) designates the two possible positions for one EcoRI cleavage site in a 2-micron DNA region which is frequently inverted in vivo (18). As the orientation of pJC75-58 was not determined, the PstI site within pJC75-58 is not included in the maps. One EcoRI site in the 9.4 kb chromosomal fragment cannot be exactly positioned from the data of Figure 2. The position of this site was determined by analysis of plasmid pC503 (Figures 5 and 6).

repeat regions give rise to equal amounts of two forms. The L form (18) is cleaved into the upper and the lower band in lane j (4.15 and 2.2 kb), whereas the R form is cleaved into the two middle bands (3.85 and 2.4 kb). Both pC500 and pC501 give 6 bands (lanes c–f), two of which they have in common (2.65 and 3.0 kb). Since pJC75-58 has one EcoRI site (4) and 2-micron DNA occur with one or two EcoRI sites (3, 17), these two fragments are suggested to be internal EcoRI fragments formed by three EcoRI sites within the chromosomal 9.4 kb PstI fragment. This leaves two EcoRI sites on the assumed 2-micron DNA part, which should give rise to an internal EcoRI fragment solely derived from 2 μ DNA. Indeed pC500 contains an EcoRI fragment (2.4 kb, lanes c and d) of the R form of the 2-micron DNA, and pC501 contains one (4.15 kb, lanes e and f) of the L form. The three remaining EcoRI fragments of pC500 (lanes c and d) have the sizes 9.5, 8.9 and 1.08 kb and those of pC501 (lanes e and f) are 6.7, 6.3 and 5.0 kb. The insertion site in pJC75-58 must be the PstI site which can be cleaved without destroying the Ap^r gene. This site is situated exactly opposite the EcoRI site in circular pJC75-58 (4).

This information completes the data needed for mapping the PstI and EcoRI sites relative to each other on pC500 and pC501, except that the orientation of pJC75-58 is unknown (Figure 3). The maps of pC500 and pC501 suggest, but do not prove, that the *HIS4* carrying yeast plasmid in C78-H26 consists of the 9.4 kb PstI fragment inserted in the PstI site of a 2-micron DNA circle. It should be noted that these two pieces of DNA have the same relative orientation in pC500 and pC501, suggesting the map for the *HIS4* carrying pure yeast plasmid given in Figure 3. We designate this plasmid pC504. A direct demonstration of the validity of the map will be given later in this paper (3.5).

3.4. Selection, propagation and further mapping of the yeast chromosomal region containing the *HIS4* gene

After the electrophoretic separation of DNA fragments according to size one may locate in the gel pattern fragments carrying particular sequences by hybridizing with radioactive DNA of complementary sequences (23). Labelled preparations of plasmids pC500 and pC501 would in this way be useful for the detection and sizing of

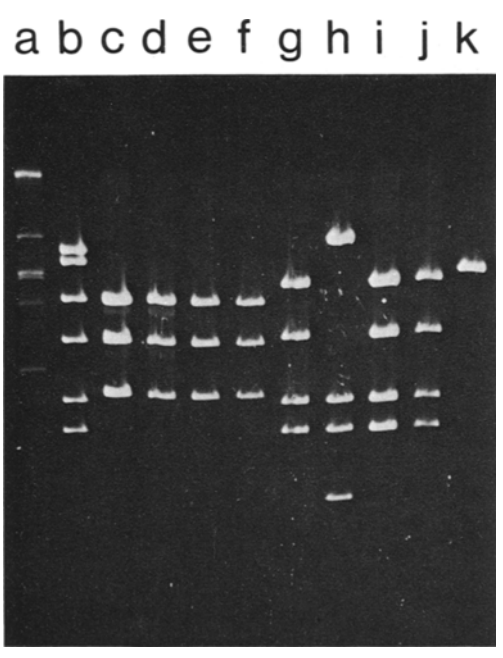


Figure 4. Screening for different insertions of yeast DNA sequences into pBR325.

Cleared lysates of E. coli clones transformed with hybrids of pC501 and pBR325 were digested with EcoRI and analysed by gel electrophoresis. EcoRI digested pC501 (lane b) and PstI digested pBR325 (lane k) were included for comparison. Lane a shows standard size markers (cf. Materials and Methods). Estimates of the fragment sizes are as follows. Lanes c–f: 5.0, 4.0 and 3.1 kb. Lanes g, i and j: 5.6, 4.1, 3.0 and 2.65 kb. Lane h: 7.7, 3.0, 2.65 and 2.0 kb. The data indicate that the plasmids in lanes c, d, e and f contain 2-micron DNA in the same orientation, those in lanes g, i and j contain the 9.4 kb PstI fragment in the same orientation whereas the plasmid in lane h contains this PstI fragment in reverse orientation.

DNA from the *HIS4* region in transformants and other strains. However, the 2-micron DNA which they contain hybridizes to the many copies of 2-micron DNA present in most samples to be analysed, obscuring the *HIS4* DNA specific hybridization. It was therefore considered important to construct a plasmid with the 9.4 kb PstI fragment but no other yeast DNA. pC501 (1.5 μg) and 6 μg of bacterial vector pBR325 (2) were treated separately with restriction endonuclease PstI to complete digestion. In order to suppress self-ligation of the linearized molecules of pBR325 they were treated with alkaline phosphatase. The digests were combined and treated with 0.2 units of DNA ligase for 48 hours. Then $5 \cdot 10^8$ cells of E. coli were transformed with this DNA and spread on plates with tetracycline, since pBR325 carries a gene for resistance to this drug. Four hundred colonies appeared, 70 of which proved by replication to be ampicillin sensitive. Since the PstI site of pBR325 is located within the gene for ampicillin resistance (2), it was assumed that each of these colonies contained a chimaeric plasmid. They were screened for plasmid size by agarose electrophoresis of cleared lysates. Eleven out of twelve fell in two size classes which by EcoRI digestion (Figure 4) were shown to represent insertion of the 9.4 kb fragment (lanes g–j) or the 2-micron DNA (lanes c–f). The plasmids represented in lanes g and h are called pC503 and pC502, respectively.

In order to map in more detail the 9.4 kb fragment, pC503 was treated with 5 different restriction enzymes and some of their pairwise combinations, and the digests analysed by electrophoresis (Figure 5). The sizes of the fragments (Table IV) led to the map shown in Figure 6. Plasmid pC502 differs from pC503 by the orientation of the insert (Figure 4, lanes g and h). The positions of the EcoRI sites in the 9.4 kb PstI fragment are the same in the four plasmids pC500 through pC503.

3.5. Characterization of the *HIS4* carrying pure yeast plasmid (pC504) in C78-H26

The only yeast sequences carried on pC502 and pC503 are the 9.4 kb fragment carrying the *HIS4* gene and they can therefore be used as hybridization probes for these particular sequences. ccDNA from C75-M26 (parent strain in the original transformation), C78-H26 (original transformant) and C78-H23/1 (retransformant), as well as plasmid pC501 were subjected to agarose gel electrophoresis before and after treatment with restriction endonucleases PstI and EcoRI. The DNA in the gel slab was transferred to a cellulose nitrate filter (23), and denatured ^{32}P-labelled pC502 DNA was hybridized to the filter. Figure 7 shows an autoradiogram of the filter. The data represented in Figure 3 lead to the expectation that four out of the six

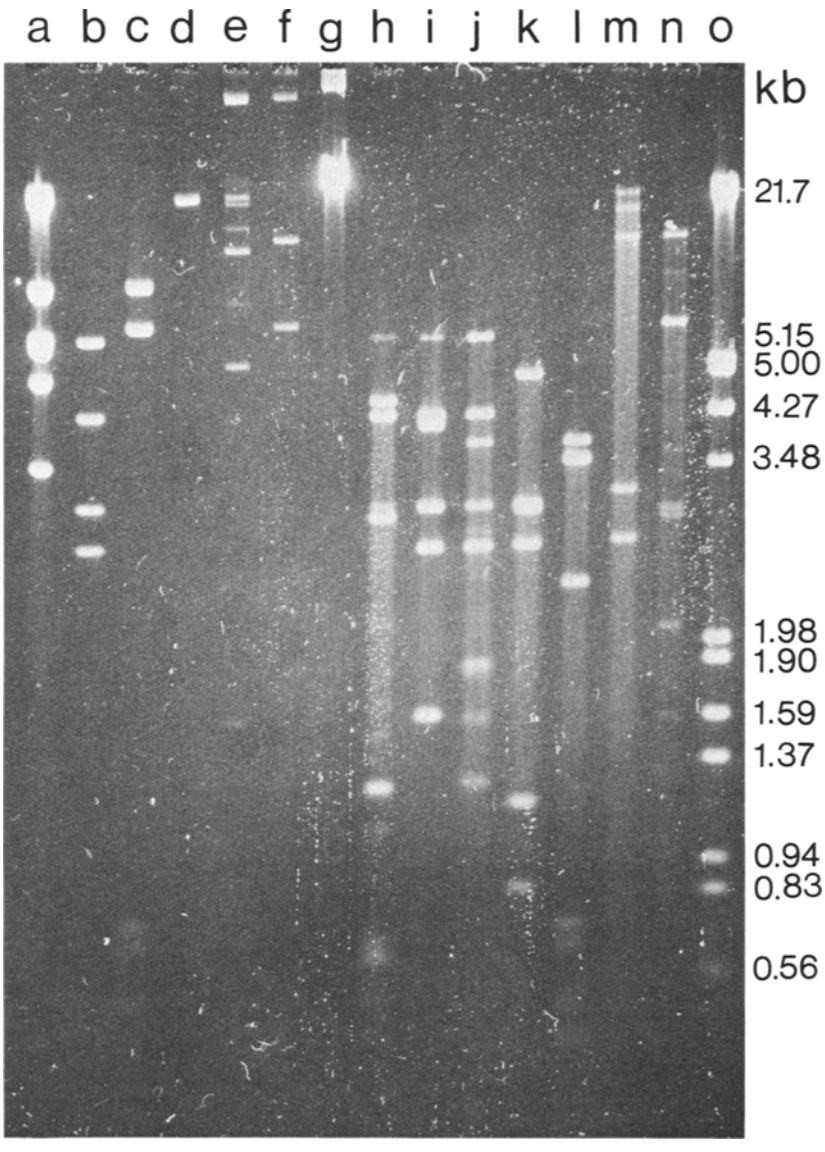


Figure 5. Cleavage analysis of pC503 with restriction endonucleases EcoRI (lane b), HindIII (lane c), BamHI (lane d), SalI (lane e) and PstI (lane f).

In addition to the single digests (lanes b–f), double digests were carried out as follows. EcoRI-HindIII (h); EcoRI-BamHI (i); EcoRI-SalI (j); EcoRI-PstI (k); PstI-HindIII (l); PstI-BamHI (m); PstI-SalI (n). Lanes a, g and o show size markers (cf. Materials and Methods). It should be noted that several bands occur which are due to incomplete digestion, especially in lanes e, j, m and n as indicated in Table IV.

EcoRI fragments of pC501 hybridize with the labelled DNA. That these four fragments indeed hybridize with pC502 is evident from Figure 7 (lane k). As expected strong hybridization also occurs to the 9.4 kb PstI fragment (lane g). In the yeast ccDNA samples, at the positions for 2-micron DNA and its fragments, a low level of unspecific binding of ^{32}P is observed, which in addition to the fragments of pC501 may serve as size markers. No other labelled bands appear in ccDNA from C75-M26 (lanes a, d and h), showing that the preparation is free from

Table IV.

Size estimates (kb) of restriction fragments of pC503 (Figure 5).

EcoRI	HindIII	BamHI	SalI	PstI	EcoRI HindIII	EcoRI BamHI	EcoRI SalI	EcoRI PstI	PstI HindIII	PstI BamHI	PstI SalI
5.6	7.3[a]	15.3[a]	oc[d]	oc[d]	5.6[b]	5.6[b]	5.6[b]	4.7	3.75	15.3[ab]	9.4[ab]
4.1	5.9		15.3[ab]	9.4[a]	4.4	4.1	4.1	3.0[c]	3.5[c]	12.4[abc]	5.9[bc]
3.0	0.69		13.8[ab]	5.9	4.1	4.0	3.7	2.65	2.36	9.4[a]	3.0
2.65	0.62		10.3[ab]		3.0[b]	3.0	3.0[b]	1.14	0.69	3.2	2.9
	0.46		8.8		2.9	2.65	2.8[b]	0.83	0.62	2.71	2.08
	0.39		6.5[b]		1.22	1.56	2.65		0.46		1.53
			5.0		0.62		1.82		0.39		
			1.53		0.59[c]		1.75[b]				
					0.46		1.53				
					0.39		1.24				

[a] calculated from other fragment sizes and found to be consistent with the band position
[b] product of incomplete cleavage
[c] assumed to be a double band
[d] open circles

chromosomal DNA. The autoradiogram patterns for C78-H26 and C78-H23/1 are identical, indicating that the structure of the plasmid is constant during growth and transformation in yeast. Cleavage with PstI reveals the 9.4 kb band (lanes e and f). With EcoRI, pC504 should give rise to seven bands, five of which should hybridize. Five labelled bands of the expected sizes were found (6.5, 5.0, 3.0, 2.65 and 1.08 kb, lanes i and j). The presence of both the 6.5 kb and the 5.0 kb bands shows that the *HIS4* carrying plasmid occurs in the yeast culture in structures corresponding to the L and R forms of 2-micron DNA, i.e. that intramolecular recombination at the inverted repeat regions takes place. According to Figure 3, the size of pC504 is 15.7 kb. This size is consistent with the position of the closed circular form of pC504 (lanes b and c) when compared to electrophoretic mobilities of ccDNA of other plasmids.

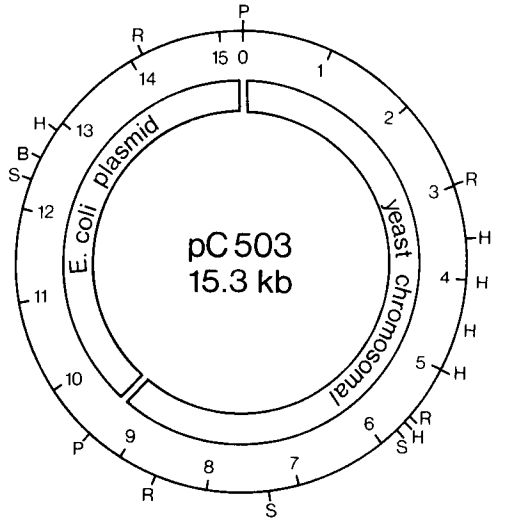


Figure 6. Cleavage map of pC503 constructed with the aid of the fragments analysed in Figure 5 and Table IV.

B, H, P, R and S designate cleavage sites for restriction endonucleases BamHI, HindIII, PstI, EcoRI and SalI, respectively. The sequence of the three small HindIII fragments between map positions 3.6 and 5 could not be determined. In the accompanying paper (13) it will be shown that the *HIS4* is located around map position 6.

4. DISCUSSION

The main objective of the present study was the molecular characterization of the *HIS4* carrying plasmid of the previously described yeast transformant C78-H26 (16). The result of the mapping of pC500 and pC501 (Figures 2 and 3) gave suggestive evidence that the *HIS4* carrying yeast plasmid is a 2-micron DNA circle with a 9.4 kb insert in the PstI site (pC504, Figure 3), but it may be argued that the 2-micron DNA present in pC500 and pC501 could have

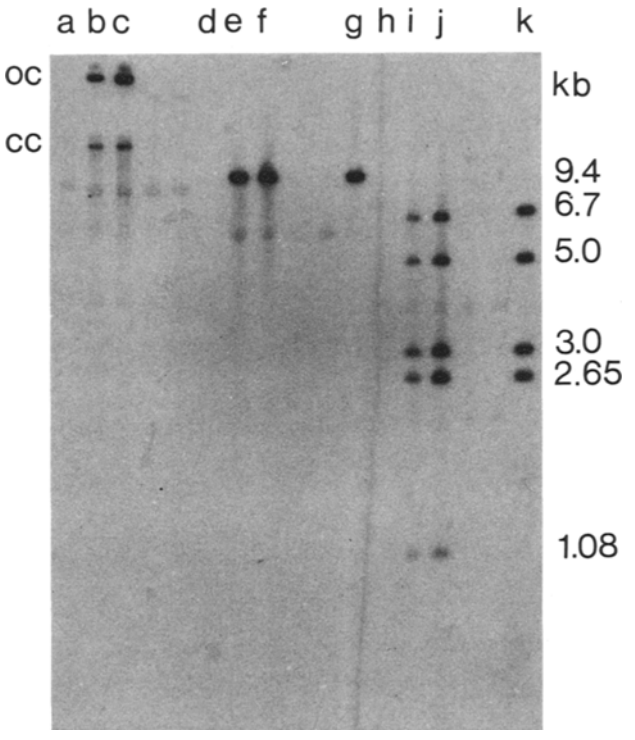


Figure 7. Electrophoretic analysis of ccDNA from yeast strains C75-M26 *(his4)*, C78-H26 (*his4*[HIS4]) and C78-H23/1 (*his4*[HIS4]).

By molecular hybridization with ^{32}P-labelled pC502 and subsequent autoradiography, fragments are detected which contain sequences occurring in the 9.4 kb chromosomal segment containing *HIS4*. Lanes a, b and c show ccDNA preparations from strains C75-M26, C78-H26 and C78-H23/1, respectively. Lanes d, e and f contain PstI digests, and lanes h, i and j contain EcoRI digests, of the same DNA preparations. PstI and EcoRI digests of plasmid pC501 were included for comparison and sizing (lanes g and k, respectively).

been inserted in vitro when the ligation to the cosmid was performed. Therefore the hybridization analysis given in Figure 7 was carried out. This experiment showed the presence in the two transformants of a yeast plasmid consisting of the 9.4 kb chromosomal DNA fragment inserted into 2-micron DNA, proving the structure given in Figure 3. The structures of pC500 and pC501 as well as the hybridization patterns (Figure 7) show that the 2-micron DNA part of pC504 occurs in two forms. BEGGS (1) reported a similar observation in the case of a plasmid consisting of the bacterial plasmid pMB9, 2-micron DNA and the yeast *LEU2* gene. We find it interesting that the 2-micron DNA part of the *HIS4* carrying yeast plasmid, pC504, contains two EcoRI sites, since the original transformation (16) was carried out with a 2-micron DNA carrying only one EcoRI site. Double digestion with PstI and EcoRI of the latter 2-micron DNA gave four bands, demonstrating that the single EcoRI site is separated from the PstI site by the inverted repeat regions. Since the two EcoRI sites in the 2-micron DNA part of pC504 are also separated by the inverted repeat regions, the new EcoRI site is on the unique sequence region which carries the PstI site. This finding indicates that recombination in 2-micron DNA is not limited to the inverted repeat regions. The map of the 2-micron DNA part of pC504 seems identical to that of the many copies of 2-micron DNA in C78-H26 (Figure 2, lane j) and its parent, C75-M26. While the use of pC504 as a pure yeast transformation vector is limited by

the difficulty of getting reasonable amounts of the pure plasmid, this study has provided us with several yeast-bacterial hybrid plasmids that may be useful as vectors for other yeast genes. The mapping of the 9.4 kb PstI fragment with four restriction endonucleases may be of help in such use of the plasmids. The accompanying paper (13) will show that the *HIS4* gene is located in a region corresponding to the lower right part of the map of pC503 shown in Figure 6.

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