JOURNAL OF BACTERIOLOGY, Sept. 1982, p. 1380–1390
0021-9193/82/091380-11$02.00/0
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Vol. 151, No. 3

2-μm DNA-Like Plasmids in the Osmophilic Haploid Yeast *Saccharomyces rouxii*

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Received 29 December 1981/Accepted 5 May 1982

DNA plasmids were detected in two independent strains of *Saccharomyces rouxii* among 100 yeast strains other than *Saccharomyces cerevisiae* tested. The plasmids, pSR1 and pSR2, had almost the same mass (approximately 4×10^6 daltons) as 2-μm DNA of *S. cerevisiae*. pSR1 and pSR2 gave identical restriction maps with restriction endonucleases *Bam*HI, *Eco*RI, *Hin*cII, *Hin*dIII, and *Xho*I, and both lacked restriction sites for *Pst*I, *Sal*I, and *Sma*I. These maps, however, differed significantly from that of *S. cerevisiae* 2-μm DNA. Restriction analysis also revealed two isomeric forms of each plasmid and suggested the presence of a pair of inverted repeat sequences in the molecules where intramolecular recombination took place. DNA-DNA hybridization between the pSR1 and pSR2 DNAs indicated significant homology between their base sequences, whereas no homology was detected between pSR1 and pJDB219, a chimeric plasmid constructed from a whole molecule of 2-μm DNA, plasmid pMB9, and a 1.2-kilobase DNA fragment of *S. cerevisiae* bearing the *LEU2* gene. A chimeric plasmid constructed with pSR1 and YIp1, the larger *Eco*RI-*Sal*I fragment of pBR322 ligated with a 6.1-kilobase DNA fragment of *S. cerevisiae* bearing the *HIS3* gene, could replicate autonomously in an *S. cerevisiae* host and produced isomers, presumably by intramolecular recombination at the inverted repeats.

The presence of an accessible DNA plasmid in an organism indicates that the organism may be useable in a host-vector system in recombinant DNA experiments. In *Saccharomyces cerevisiae*, most haploid strains harbor 50 to 100 copies of 2-μm DNA plasmid per cell (8). 2-μm DNA exists as a histone-bound chromosome, as does nuclear chromatin (25, 31). Although various restriction maps of 2-μm DNA have been observed depending on the yeast strain (T. Oshima and I. Takano, personal communication), their gross molecular structure is characterized (13, 14, 17, 20, 26, 35) by closed circular DNA with a mass of 4×10^6 daltons and a size of 6×10^3 base pairs, by a pair of approximately 600-base pair inverted repeats, and by two isomeric forms due to the intramolecular recombination at the inverted repeats. The entire nucleotide sequence of 2-μm DNA harbored by an *S. cerevisiae* strain, A364A D5, has been determined (17), and three possible long open translation reading frames were found in the molecule. 2-μm DNA has been proved to share, at least in part, the same replication mechanisms as nuclear DNA (27, 32, 44, 50), although the two DNAs show a striking difference in copy numbers. The replication origin has been delimited to a region of several hundred base pairs, including part of one of the inverted repeats (5). This recent accumulation of knowledge on 2-μm DNA makes this plasmid a promising and sophisticated system for investigation of the DNA replication mechanism (22) as well as for application in recombinant DNA experiments as a vector for yeast hosts.

Except for mitochondrial DNA, extrachromosomal DNAs have rarely been reported in yeasts or fungi other than *S. cerevisiae*. The rare examples are a 2-μm DNA-like plasmid in *Schizosaccharomyces pombe* (12) and a 3×10^6 dalton DNA plasmid in *Podospora anserina* (38). Recently, Gunge et al. (16) isolated two species of linear DNA plasmids from a strain of *Kluyveromyces lactis*. One or both of these plasmids might be connected with the killer phenomenon of this yeast.

This communication describes two plasmids, pSR1 and pSR2, detected in independent strains of *Saccharomyces rouxii* among 100 yeast strains so far tested belonging to 10 species in 9 genera. The *S. rouxii* plasmids both have almost the same size as 2-μm DNA and seem to contain a pair of inverted repeat sequences. No differences have yet been found between the two *S.*

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rouxii plasmids, which are, however, entirely different molecules from 2-μm DNA. Plasmid pSR1 may have a replication origin effective in an *S. cerevisiae* host.

MATERIALS AND METHODS

Organisms and plasmids. The principal yeast strains, bacterial strains, and plasmids used in this study are listed in Table 1, including the two strains of *S. rouxii* (G4118 and IFO 1130) in which plasmids were detected. The following 98 strains of yeasts other than *S. cerevisiae* are those in which plasmids were not be detected: *Candida albicans* (IFO 1386, IFO 1388, IFO 1594), *Candida tropicalis* (IFO 1401, IFO 1402, IFO 1403, pK233, ATCC 22577), *Debaryomyces hansenii* (IFO 0017, IFO 0018, IFO 0019, IFO 0023, IFO 0026, IFO 0037, IFO 0042, IFO 0047, IFO 0058, IFO 0059, IFO 0066, IFO 0083, OUT 6035, OUT 6036, OUT 6046, OUT 6048, OUT 6052, OUT 6053), *Hansenula anomala* (NRRL 1798, IFO 0131, OUT 6081, OUT 6084, OUT 6091, OUT 6093, OUT 6232, OUT 6234, OUT 6235), *Pichia membranaefaciens* (OUT 6124, OUT 6126, OUT 6128, OUT 6129, OUT 6130, OUT 6131, OUT 6132, OUT 6134, OUT 6135, OUT 6137, OUT 6139, OUT 6140, OUT 6141, OUT 6236, OUT 6237, OUT 6240, OUT 6286, OUT 6287, OUT 6298), *S. rouxii* (IFO 0487, IFO 0495, IFO 0533, IFO 0686, IFO 0740, IFO 1252, IFO 1812, IFO 1813, IFO 1814, 019-5, G4106, A31, N28, 510, 1028, NRRL 2547, NRRL 2548), *Saccharomycodes ludwigii* (ATCC 26617, ATCC 26618, O-16 [ATCC 44294], O-17 [ATCC 44295], O-18 [ATCC 44296], O-59 [ATCC 44297], O-61 [ATCC 44298], O-62 [ATCC 44299], O-80 [ATCC 44300], O-81 [ATCC 44301], IFO 0798, IFO 1043-1, IFO 1043-2, IFO 1194, IFO 1266, M-1, M-2), *Saccharomycopsis lipolytica* (MK4), *S. pombe* (IFO 0347, IFO 0348, IFO 0349, OUT 6175, OUT 6177, L972, L975), and *Trichosporon cutaneum* (OUT 6259, OUT 6308).

C. tropicalis strain pK233 was obtained from M. Osumi of the Department of Biology, Japan Women's University, Tokyo. Nine strains of *S. rouxii* (those not bearing IFO code numbers) were supplied by H. Mori of the Central Research Laboratories of Kikkoman Co. Ltd., Chiba, Japan. Two of the strains of *S. ludwigii*, M-1 and M-2, were obtained from T. Yamazaki of the Department of Fermentation Technology, Yamanashi University, Yamanashi, Japan; the remainder were those described previously (49). *S. lipolytica* MK4 was supplied by H. Matsuoka of the Department of Fermentation Technology, Osaka University, Osaka, Japan, and *S. pombe* strains L972 and L975 were from C. Shimoda of the Department of Biology, Osaka City University, Osaka, Japan. The other yeast strains were obtained from the Institute for Fermentation, Osaka (the strains bearing the IFO code), the American Type Culture Collection, Rockville, Md. (ATCC code), Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Ill. (NRRL code), or selected from our culture collection (OUT code).

S. rouxii is an osmophilic yeast which grows well in highly concentrated NaCl or sugar medium (47). Its life cycle has a dominant vegetative phase with haploid cells of **a** or α mating type (29). Although these cells resemble haploid cells of *S. cerevisiae*, the two yeasts cannot be interbred.

TABLE 1. Principal microorganisms and plasmids

Organism or plasmid	Genotype or characteristics or both[a]	Source or reference
S. rouxii G4118	Wild type	H. Mori
S. rouxii IFO 1130	Wild type	Institute for Fermentation, Osaka
S. cerevisiae D13-1A	*MATa trp1 his3* [*cir*$^+$]	(41)
E. coli JA221	F$^-$ *leuB6 ΔtrpE5 lacY hsdR*$^-$ *hsdM*$^+$ *recA*	(7)
YIp1	Amp^r *HIS3* (6.5×10^6)	(40, 41)
YRp7	Amp^r Tet^r *TRP1* (3.7×10^6)	(41)
pJDB219	Tet^r *LEU2* (8.0×10^6)	(1)
pBR322	Amp^r Tet^r (2.8×10^6)	(4) (42)
pSR1	Cryptic (4.0×10^6)	This study
pSR2	Cryptic (4.0×10^6)	This study
pSRT1	Amp^r *HIS3* (8.8×10^6)[b]	This study
pSRT2	Amp^r *HIS3* (8.8×10^6)[b]	This study

[a] The genetic symbols for yeasts are those proposed by the Nomenclature Committee for Yeast Genetics (33), and [*cir*$^+$] indicates the presence in cells of 2-μm DNA. Genetic symbols for *E. coli* follow Taylor (46), and *hsdR*$^-$ and *hsdM*$^+$ indicate defective host-specific restriction and active host-specific modification, respectively. Amp^r and Tet^r indicate the phenotypes of ampicillin and tetracycline resistance, respectively. Numbers in parentheses are plasmid masses in daltons.

[b] Calculated from the data of Struhl et al. (41), Struhl and Davis (40), Boliver et al. (4), Sutcliffe (42), and the present study.

Media. YPAD medium (10 g of yeast extract [Daigo Eiyo Chemicals, Osaka, Japan], 20 g of Polypepton [Daigo Eiyo Chemicals], 400 mg of adenine, and 20 g of glucose per liter) or YPG medium (20 g of yeast extract, 20 g of Polypepton, and 30 g of glycerol per liter) was used as a nutrient medium for the cultivation of yeast strains. Minimal medium for yeast contained 6.7 g of Yeast Nitrogen Base without amino acids (Difco Laboratories, Detroit, Mich.) and 20 g of glucose per liter. Amino acids or nucleic acid bases were added appropriately. For cultivation of *Escherichia coli*, nutrient broth (Difco) and L broth (10 g of tryptone [Difco], 5 g of yeast extract [Difco], and 5 g of NaCl) were used. Amino acid (40 μg/ml), uracil (40 μg/ml), thiamine (1 μg/ml), Viccillin (sodium ampicillin [50 μg/ml]; Meiji Seika, Tokyo, Japan) or tetracycline (10 μg/ml; Sigma Chemical Co., St. Louis, Mo.) was supplemented when necessary. Solid media were prepared by adding 2% of agar. Yeasts were cultivated at 30°C, and *E. coli* at 37°C.

Transformation. Competent cells of *E. coli* were prepared by $CaCl_2$ treatment (28) and stored in glycerol at −80°C until use (30). *E. coli* transformation with either purified or crude DNA preparation was carried out according to the method described by Lederberg and Cohen (24). For transformation of yeast cells, the procedure of Beggs (1) was followed, except that Zymolyase 60000 (Kirin Brewery Co., Tokyo, Japan) at 0.1 μg/ml was used instead of Helicase.

Preparation of DNA. For detection of plasmid DNA in yeast, cells grown in 5 ml of YPAD were converted to protoplasts with Zymolyase 60000 by the method of Cameron et al. (6) for all yeast strains except those of *C. albicans*, *C. tropicalis*, *S. pombe*, and *S. lipolytica*. The exceptional strains were cultivated in YPG (*S. pombe*) or minimal medium (*Candida* spp. and *S. lipolytica* strains) and converted to protoplasts by the method of Foury and Goffeau (11) for *S. pombe* or of Stahl (37) for *Candida* spp. and *S. lipolytica* strains. Lysates were prepared from the protoplasts as described by Cameron et al. (6). The precipitates that formed upon the addition of two volumes of ethanol (95%) to these lysates were dissolved in a minimal amount of 10 mM Tris-hydrochloride buffer with 1 mM EDTA (pH 7.5) containing heat-treated RNase A (5 μg/ml; Sigma) and incubated at 37°C for 1 h. These solutions were then subjected to agarose gel electrophoresis.

Yeast plasmids were isolated by essentially the same procedure as that described by Livingston and Klein (26). In brief, the supernatant fraction was prepared by the method of Hirt (19) from the spheroplasts of cells cultivated in 1 liter of YPAD medium by shaking at 30°C for 24 h. Nucleic acids were precipitated with 10% polyethylene glycol 6000 (21), redissolved in the Tris-EDTA buffer containing 1 M NaCl, and the insoluble material was removed after overnight incubation at 0°C. The resulting supernatant (approximately 40 ml) was distributed into four centrifuge tubes to which 12 g of CsCl, 2 ml of ethidium bromide solution (10 mg/ml), and Tris-EDTA buffer were added to bring the weight of liquid to 27.2 g. The mixture was centrifuged at 35,000 rpm for 48 h at 18°C in a Hitachi RP50-2 rotor. Fractions containing closed circular DNA were removed with a syringe inserted through the side of the centrifuge tube. The pooled fractions were centrifuged again in a Hitachi RP65 rotor at 35,000 rpm for 48 h at 18°C and the DNA was collected with a syringe as described above. The DNA fraction containing plasmids was washed with isoamyl alcohol to remove ethidium bromide, dialyzed against the Tris-EDTA buffer at 4°C, and the DNA was precipitated by the addition of two volumes of 95% ethanol. DNA was collected by centrifugation at 12,000 rpm for 20 min in a Sorvall SS34 rotor and then dissolved in a small amount of the Tris-EDTA buffer to a concentration of 50 to 100 μg/ml.

For preliminary characterization of plasmids extracted from the bacterial host, DNA was prepared by the rapid alkali extraction method of Birnboim and Doly (3). Since all of the bacterial plasmids used in this work are derivatives of ColEl, plasmid DNA was amplified by the addition of chloramphenicol to the medium (100 μg/ml) (9) when the optical density of the culture reached 0.5 to 0.6 units at 660 nm followed by further incubation at 37°C with shaking for 20 h. Purified bacterial plasmid DNA was prepared from cleared lysate (10) by CsCl-ethidium bromide gradient centrifugation in a Hitachi RP65 rotor at 35,000 rpm for 40 h at 18°C.

Enzymes. Restriction endonucleases were purchased from Takara Shuzo Co. Ltd., Kyoto, Japan, and were used in the buffers recommended by the manufacturer. The amount of DNA in each reaction mixture (50 μl) was 0.5 to 1 μg. T4 ligase was prepared in our laboratory by the procedure of Weiss (48). Ligation mixture was prepared according to Tanaka and Weisblum (45).

Agarose gel electrophoresis. Slab gels (15 by 18 by 0.3 cm) were prepared by pouring melted 1% agarose. Electrophoresis was carried out at 2 V per cm overnight or 5 V per cm for 4 h. DNA bands were stained with ethidium bromide and photographed under long-wave (365 nm) UV light. λ DNA purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., was used as a size marker (43) in the gel electrophoresis after digestion with *Hin*dIII.

Nick translation and Southern hybridization. Transfer of DNA from an agarose gel to a nitrocellulose sheet (PKG SMWPO 00 10; Millipore Corp. Bedford, Mass.) by the method of Southern (36) and DNA hybridization were performed as described by Jeffreys and Flavell (23) at 65°C for 18 h in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.2% Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N.J.), 0.2% polyvinylpyrrolidone (Wako Pure Chemical, Osaka, Japan), 0.2% bovine serum albumin (Miles Laboratories, Inc., Elkhart, Ind.), 50 μg of denatured sonicated calf thymus DNA (Miles) per ml, and 0.1% sodium dodecyl sulfate. Two probes were employed: the whole molecule of pJDB219 plasmid (1), and the pSR1 plasmid from *S. rouxii* described below. ^{32}P-labeled probes were prepared by nick translation as described by Rigby et al. (34). Specific radioactivities of the probes were approximately 10^7 cpm per μg of DNA.

RESULTS

DNA plasmids in *S. rouxii*. DNA prepared from each of the 100 yeast strains was analyzed by agarose gel electrophoresis. Most strains showed only one heavy DNA band corresponding to nuclear and mitochondrial DNAs, whereas two strains of *S. rouxii*, G4118 and IFO 1130, each showed two extra bands (Fig. 1A). For comparison, DNA was similarly prepared from *S. cerevisiae* D13-1A and run on an agarose gel parallel to those of *S. rouxii*. Each of the satellite DNA bands of *S. rouxii* can be superimposed on bands corresponding to the open and closed circular forms of 2-μm DNA of *S. cerevisiae* (Fig. 1B). This indicates that these two strains of *S. rouxii* carry 2-μm DNA-like plasmids. Comparison of the intensity of fluorescence of the bands on the gel indicates that the copy numbers of the plasmids in *S. rouxii* are roughly the same as that of 2-μm DNA in *S. cerevisiae*. The plasmids of strains G4118 and IFO 1130 were designated pSR1 and pSR2, respectively.

To characterize these plasmids further, the

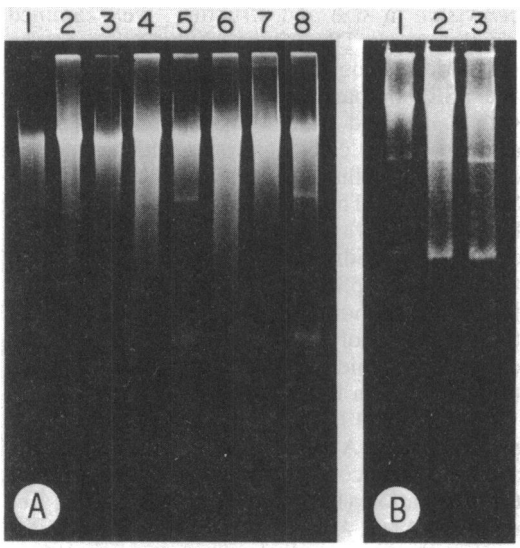

FIG. 1. Agarose gel electrophoresis of crude DNA extracts of *S. rouxii* and *S. cerevisiae*. DNAs prepared from strains of *S. rouxii* or *S. cerevisiae* by the rapid method described by Cameron et al. (6) were electrophoresed on 1% agarose gel. (A) DNAs from *S. rouxii* strains IFO 0487 (lane 1), IFO 0495 (lane 2), IFO 0533 (lane 3), IFO 0740 (lane 4), IFO 1130 (lane 5), IFO 1812 (lane 6), IFO 1813 (lane 7), and G4118 (lane 8). (B) DNAs from *S. cerevisiae* D13-1A (lane 1), *S. rouxii* IFO 1130 (lane 2), and *S. rouxii* G4118 (lane 3).

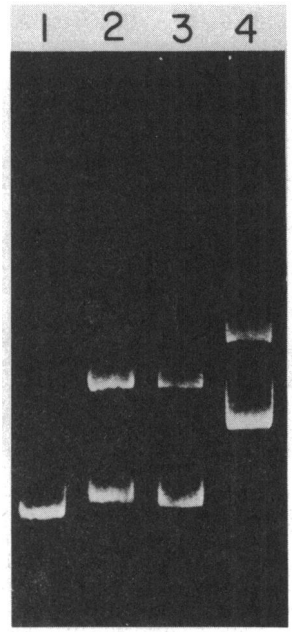

FIG. 2. Estimation of the masses of pSR1 and pSR2 by relative mobility of the plasmids on agarose gel. Lane 1, YRp7 (3.7×10^6 daltons); lane 2, pSR1; lane 3, pSR2; and lane 4, YIp1 (6.5×10^6 daltons).

plasmid DNAs were purified by CsCl-ethidium bromide density gradient centrifugation. These were electrophoresed with purified plasmid DNA from YRp7 (3.7×10^6 daltons) (41) and YIp1 (6.5×10^6 daltons) (41), and their molecular sizes were both estimated from their relative electrophoretic mobility to be approximately 4×10^6 daltons (Fig. 2). The circularity of the pSR1 plasmid was confirmed by electron microscopic observation (not shown). The contour length of pSR1 DNA was approximately 2 μm on the average, and its mass was calculated as 4×10^6 daltons against open circular pBR322 (2.8×10^6 daltons) (42) as reference (data not shown).

Restriction analysis of pSR1 and pSR2. Purified pSR1 and pSR2 DNAs were subjected to restriction analysis (Fig. 3). Each DNA has a unique site for *Hin*dIII and *Xho*I, and each has two sites for *Bam*HI and *Eco*RI. Since one of the two *Eco*RI fragments gave a faint band (which was visible on an overexposed photograph although not seen on Fig. 3) at the site corresponding to 0.15×10^6 daltons, the two *Eco*RI sites were situated close to each other. For *S. cerevisiae*, *Bam*HI and *Xho*I sites have not been found for the 2-μm DNA sequence in 15 independent plasmids so far tested (T. Oshima and I. Takano, personal communication). The digestion of pSR1 and pSR2 with *Hin*cII gave rise to six fragments, of which the smallest was visible only on an overexposed photograph. These plasmids could not be cut with *Sal*I, *Sma*I, or *Pst*I, although *Pst*I cuts 2-μm DNA at one site. When pSR1 and pSR2 were digested with a combination of *Eco*RI and *Hin*dIII or *Eco*RI and *Xho*I, five bands appeared on the gel (Fig. 4), the smallest of which was very faint and hardly visible on the photograph. The sum of the masses of the four major bands was approximately 8×10^6 daltons, whereas the sum of the largest and the fourth-largest ones and of the second-largest and third-largest ones were almost equal at approximately 4×10^6 daltons. These observations indicate two isomeric forms of DNA in pSR1 and pSR2, as in 2-μm DNA of *S. cerevisiae*, and strongly suggest the presence of a pair of inverted repeat sequences in these DNA molecules. The molecular sizes of restriction fragments were estimated by comparison with the fragments of phage λ DNA digested with *Hin*dIII (43) and electrophoresed in parallel (Table 2). The results indicated a molecular size of approximately 4×10^6 daltons for the pSR1 DNA (and also for the pSR2 DNA, the data for which are omitted in Table 2). Analysis with these restriction endonucleases thus indicates that the two plasmids have the same DNA sequences as each other. This conclusion is

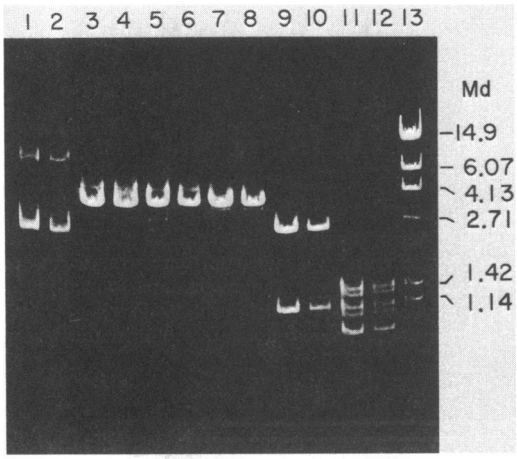

FIG. 3. Agarose gel electrophoresis of single-enzyme digests of the pSR1 and pSR2 DNAs. Undigested pSR1 and pSR2 DNAs were run on lanes 1 and 2, respectively. Lanes 3, 5, 7, 9, and 11 are pSR1 digested with *Eco*RI, *Hin*dIII, *Xho*I, *Bam*HI, and *Hin*cII, respectively. Lanes 4, 6, 8, 10, and 12 are pSR2 digested with *Eco*RI, *Hin*dIII, *Xho*I, *Bam*HI, and *Hin*cII, respectively. Lane 13 is the λ DNA digested with *Hin*dIII, and the approximate fragment sizes in megadaltons (Md) are indicated in the right-hand margin.

supported by DNA-DNA hybridization between pSR1 and pSR2, as shown below.

The pSR1 molecule may be separated into two unique sequences by the inverted repeats; one sequence carries the two closely linked *Eco*RI sites and the other the *Xho*I and *Hin*dIII sites. Intramolecular recombination in the inverted repeats results in two forms of pSR1. However, *Bam*HI digestion generates two fragments of 2.7 $\times 10^6$ and 1.2 $\times 10^6$ daltons (Fig. 3 and 4, and Table 2), rather than four. The *Bam*HI sites must therefore be located in the inverted repeats or at sites equidistant from the two inverted repeats on one of the unique sequences. The restriction map (Fig. 5) of the pSR1 DNA was constructed with data for *Eco*RI, *Hin*dIII, *Bam*HI, *Xho*I, and for combinations of two of the endonucleases (Table 2). The two faint bands situated on each side (upper and lower) of the main bands in the gel patterns of the DNA digested with *Eco*RI, *Xho*I, or *Hin*dIII (Fig. 4) can be explained by this structure as having resulted from the cleavage of dimers of the pSR1 molecule, similar to the phenomenon observed with 2-μm DNA in *S. cerevisiae*. The restriction sites for *Hin*cII are unknown, as we did not analyze the positions of the six *Hin*cII sites relative to the other restriction sites. The same map should also hold for pSR2.

pSR1 DNA homology with 2-μm DNA. Since pSR1 and pSR2 resemble 2-μm DNA of *S. cerevisiae* in size and structure, we examined whether these DNA species share a common nucleotide sequence. DNA fragments of pSR1 and pSR2 prepared by simultaneous digestion with *Eco*RI and *Hin*dIII were separated on an agarose gel by electrophoresis and transferred onto a nitrocellulose sheet by the method of Southern (36). The ^{32}P-labeled pSR1 DNA probe hybridization was carried out. Almost the same extent of hybridization was observed for both the pSR1 and pSR2 DNA fragments (Fig. 6A). However, no homology was detected between the fragments of pSR1 DNA and those of the chimeric plasmid pJDB219 (1), which contains the whole 2-μm DNA molecule (Fig. 6B).

Possible pSR1 DNA replication origin for the *S. cerevisiae* host. A plasmid DNA which functions as a yeast replicon results in a high frequency of transformation (10^3 or more transformants per μg of DNA) (1, 41), whereas one lacking a yeast replicon transforms in lower frequency (10 or fewer transformants per μg of DNA) (18). The criterion of transformation frequency allowed detection in the pSR1 DNA of an autonomously replicating sequence effective in *S. cerevisiae* host. YIp1 (41) is a chimeric plasmid constructed by ligation of the larger *Eco*RI-*Sal*I fragment of pBR322 (4, 42) and a 6.1-kilobase DNA fragment of *S. cerevisiae* carrying the *HIS3* gene (40) and has restriction sites for *Bam*HI, *Eco*RI, *Hin*dIII, *Sal*I, and *Xho*I, as illustrated in Fig. 5. Since it does not contain a replication origin for yeast, it transforms a *his3* yeast host to the His$^+$ phenotype only in a low frequency (41). A hybrid plasmid between YIp1 and pSR1 was constructed and tested for its ability to transform a *his3* host to the His$^+$ phenotype. The pSR1 DNA digested with *Xho*I was mixed with YIp1 DNA digested with *Sal*I and *Xho*I, and they were ligated with T4 ligase. The ligated DNA was introduced into *E. coli* cells of strain

TABLE 2. Size of restriction fragments of pSR1[a]

Enzyme	Fragment mass (10^6 daltons)					
	1	2	3	4	5	6
*Bam*HI	2.70	1.20				
*Eco*RI	3.80	0.15				
*Hin*cII	1.50	1.40	1.20	1.10	0.95	0.25
*Hin*dIII	3.95					
*Xho*I	3.95					
*Eco*RI + *Bam*HI	1.85	1.20	0.75	0.15		
*Eco*RI + *Hin*dIII	2.75	2.05	1.70	1.00	0.15	
*Eco*RI + *Xho*I	2.70	2.40	1.45	1.10	0.15	
*Bam*HI + *Hin*dIII	2.70	0.95	0.25			
*Bam*HI + *Xho*I	2.70	0.80	0.40			
*Hin*dIII + *Xho*I	3.85	0.15				

[a] Essentially the same results were obtained with the pSR2 DNA.

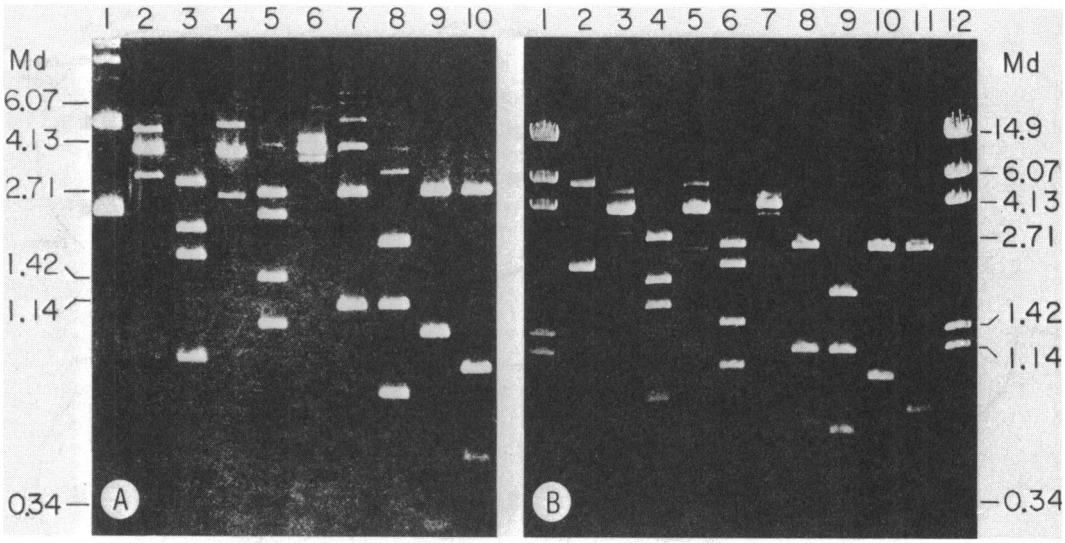

FIG. 4. Agarose gel electrophoresis of pSR1 and pSR2. (A) pSR1 was digested with the following enzymes: lane 1, none; lane 2, *Hin*dIII; lane 3, *Eco*RI and *Hin*dIII; lane 4, *Eco*RI; lane 5, *Eco*RI and *Xho*I; lane 6, *Xho*I; lane 7, *Bam*HI; lane 8, *Bam*HI and *Eco*RI; lane 9, *Bam*HI and *Hin*dIII; and lane 10, *Bam*HI and *Xho*I. Approximate positions of the fragments of λ DNA are indicated in the left-hand margin along with their masses in megadaltons (Md). (B) pSR2 was digested with the following enzymes: lane 2, none; lane 3, *Hin*dIII; lane 4, *Eco*RI and *Hin*dIII; lane 5, *Eco*RI; lane 6, *Eco*RI and *Xho*I; lane 7, *Xho*I; lane 8, *Bam*HI; lane 9, *Bam*HI and *Eco*RI; lane 10, *Bam*HI and *Hin*dIII; and lane 11, *Bam*HI and *Xho*I. Lanes 1 and 12 are λ DNA digested with *Hin*dIII, and the approximate fragment masses in megadaltons (Md) are indicated in the left-hand margin.

JA221, and transformants showing the Amp^r phenotype were selected. Then a plasmid larger than YIp1 was sought in the transformants by rapid alkaline extraction of DNA (3) and agarose gel electrophoresis. One such plasmid, pSRT1, was found among 100 Amp^r *E. coli* transformants tested.

Since *Xho*I and *Sal*I produce the same sticky ends and the pSR1 DNA is a mixture of two isomers, pSRT1 DNA can have any of the four structures (1A, 1B, 2A, and 2B) illustrated in Fig. 5. To determine the structure of pSRT1, the DNA was purified by CsCl-ethidium bromide centrifugation, digested with *Eco*RI, *Xho*I, *Hin*dIII, or *Bam*HI, or combinations of these enzymes, and then analyzed by agarose gel electrophoresis, with the pSR1 and YIp1 DNAs digested in the same way as the reference DNA (Fig. 7). It should be borne in mind that one of the joints, *Sal*I/*Xho*I, is not cleaved by either *Sal*I or *Xho*I. Since pSRT1 contained three *Eco*RI and one *Xho*I site as predicted from the construction scheme (Fig. 5), four fragments were expected by double digestion with *Eco*RI and *Xho*I. However, only three bands were visible on the gel (Fig. 7, lane 5), as the two *Eco*RI sites on pSR1 are situated close to each other (Fig. 5), and the smallest *Eco*RI fragment generated by the digestion is hardly visible. The third band from the top of lane 7 had the same mobility as that of the smaller fragment, which is 2.3×10^6 daltons in mass and contains the *HIS3* gene, of the YIp1 DNA by the same *Eco*RI-*Xho*I double digestion (Fig. 7, lane 6). The second band from the top of lane 5 had the same mobility as that of the largest fragment (2.7×10^6 daltons) generated by the same double digestion of the pSR1 DNA (Fig. 7, lane 4). This fact indicated that the pSRT1 DNA was constructed with the pSR1 DNA of the form A configuration (Fig. 5). Thus, the largest band of lane 5 was deduced to be the *Eco*RI-*Xho*I DNA fragment containing the *Sal*I/*Xho*I joint (Fig. 5). The second band from the top of the pSRT1 DNA cleaved by *Bam*HI (Fig. 7, lane 11) showed the same mobility as that of the larger *Bam*HI fragment of pSR1 (Fig. 7, lane 10). These results agreed with the predictions for the 1A form of the pSRT1 molecule, as illustrated in Fig. 5. However, the expected fraction of 1.0×10^6 daltons by the double digestion with *Eco*RI and *Hin*dIII was missing in the pSRT1 DNA (Fig. 7, lane 8), whereas the largest fragment in the same digest was the same size as the largest *Eco*RI-*Xho*I fragment of the same plasmid (Fig. 7, lane 5). These results suggest that the *Hin*dIII site of the pSR1 portion in pSRT1 was removed or modified during the construction of pSRT1. The restriction map of pSRT1 thus obtained is shown in Fig. 5.

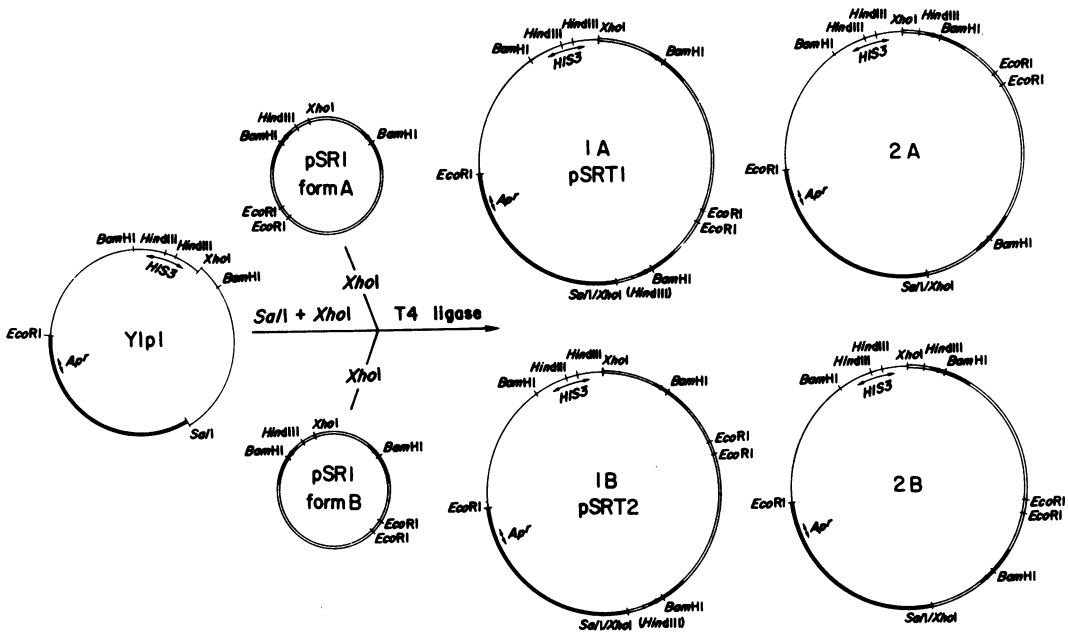

FIG. 5. Schematic illustration of plasmids. The heavy lines indicate sequences corresponding to the larger *Eco*RI-*Sal*I fragment of pBR322. The thin lines are the DNA fragments of *S. cerevisiae* containing the *HIS3* gene. The double line indicates the pSR1 DNA and the heavy lines with arrow heads are the regions for the putative inverted repeats of pSR1. The *HIS3* region and the restriction sites with *Bam*HI and *Hin*dIII on YIp1 were rewritten from Struhl and Davis (40). *Ap^r* is the coding region for the Amp^r phenotype. Since the pSR1 plasmid has two isomeric forms, A and B, four different forms, 1A, 1B, 2A, and 2B, of the chimeric plasmid composed of pSR1 and YIp1 can be expected. The *Hin*dIII site of pSRT1 or pSRT2 indicated in parentheses might be eliminated or modified during the construction of pSRT1, as described in the text.

Transformation of a *his3* mutant, D13-1A, to the His$^+$ phenotype was tested by using pSRT1 and YIp1 as donor DNAs. Protoplasts prepared from the cells of 5 ml of D13-1A culture in YPAD were mixed with 1 μg of the donor DNA and processed according to the procedure of Beggs (1). A 1-μg amount of the YIp1 DNA gave rise to four His$^+$ transformants. In contrast, 1 μg of the pSRT1 DNA gave rise to 598 and 2,016 His$^+$ transformants in different experiments under the same conditions. Since this increase in transformation frequency is significant, it is possible to conclude that the pSRT1 plasmid contains a replication origin effective for the *S. cerevisiae* host, which might be derived from pSR1.

To determine whether the His$^+$ transformants harbor pSRT1 in the plasmid state, the stability of the His$^+$ trait of the transformants was tested. Five independent yeast His$^+$ transformants by pSRT1 were grown for approximately 12 generations in YPAD or in minimal medium without addition of histidine and then spread on YPAD plates after appropriate dilution to generate about 100 colonies per plate. The His phenotype of each colony on the plates after incubation at 30°C for 2 days was then scored by replica plating the colonies onto His test plates. Mitotic segregation of His$^-$ was higher in the culture on YPAD (52 to 98%) than in that on minimal medium (2 to 35%), although the His$^-$ frequency differed significantly from one culture to another.

To further confirm the plasmid state of pSRT1 in *S. cerevisiae* cells, we tried to recover the pSRT1 plasmid from the yeast His$^+$ transformants. Each of five independent His$^+$ transformants was grown in 100 ml of YPAD. Crude DNA was prepared from each culture by the rapid method of Cameron et al. (6) and deproteinized by shaking with an equal volume of a 1:1 (vol/vol) chloroform-phenol mixture saturated with Tris-EDTA buffer. DNAs thus prepared were used as donors to transform the *E. coli* host, JA221, from Amp^s to Amp^r. Two to eight transformants, eighteen in total, were isolated for each DNA preparation. Plasmid DNA was prepared from each of the 18 transformants by alkali extraction (3), and the size of the plasmid DNA in each transformant was compared with that from pSRT1 by agarose gel electrophoresis. Plasmids of the same size as pSRT1 were recovered from 16 of the 18 transformants, whereas the remaining 2 transformants harbored smaller

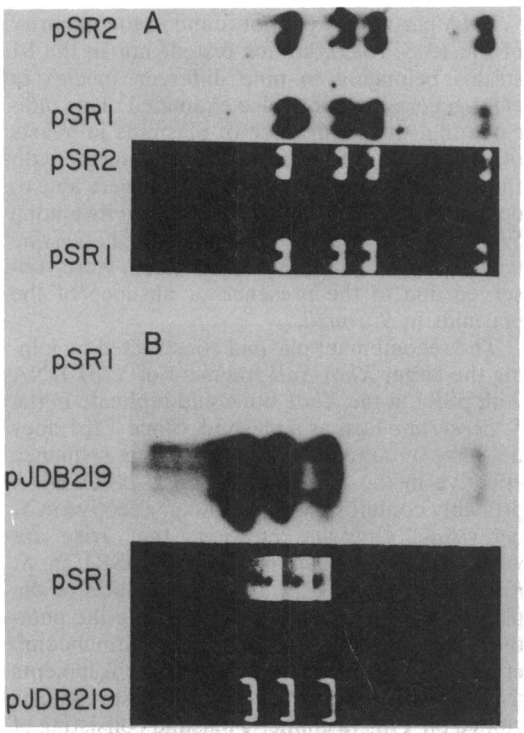

FIG. 6. Hybridization of DNA fragments of pSR1, pSR2, and pJDB219 with ^{32}P-labeled pSR1 or pJDB219 DNA as probe. (A) pSR1 and pSR2 DNAs digested with *Eco*RI and *Hin*dIII were run on 1% agarose gel, transferred to a nitrocellulose sheet and hybridized with ^{32}P-labeled pSR1 DNA. The sheet was then washed six times at 65°C with hybridization medium without the probe DNA and once with 3× SSC at room temperature and then autoradiographed at −80°C with Kodak X-Omat film for an appropriate time period. (B) pJDB219 DNA digested with *Eco*RI and pSR1 DNA double digested with *Eco*RI and *Hin*dIII were electrophoresed on agarose gel and hybridized with ^{32}P-labeled pJDB219 DNA as the probe by the same procedure as that described for (A).

plasmids (by approximately 2 × 10^6 daltons). In contrast, no transformants were obtained with DNA prepared from the original strain of D13-1A. These observations clearly indicated that pSRT1 replicated in the *S. cerevisiae* host in the plasmid state. Therefore, we concluded that the pSRT1 DNA, and hence the pSR1 DNA, has a sequence which confers on the plasmids the ability to replicate in the *S. cerevisiae* host.

Intramolecular recombination of pSRT1 in the *S. cerevisiae* host. Plasmids of the same size as pSRT1 were purified by CsCl-ethidium bromide gradient centrifugation from the seven independent *E. coli* Amp^r transformants described above. When each plasmid DNA digested with *Eco*RI and *Xho*I was run on agarose gel, we observed two different groups of DNAs. In four cases, the gel patterns were the same as that of pSRT1, whereas three showed a different pattern. One of the plasmids showing the different pattern, designated pSRT2, was compared with the pSRT1 and pSR1 DNAs after double digestion with *Eco*RI and *Xho*I by running their digests in parallel on agarose gel (Fig. 8). The third band from the top of pSRT2 showed the same migration distance as the third band of pSR1, which would have resulted from similar digestion of pSR1 of form B (Fig. 5); the second band of pSRT2 showed the same migration as the third band of pSRT1, and the largest fragment of pSRT2 was much larger than the largest fragment of pSRT1 DNA. These facts indicated that the pSRT2 was an isomer of pSRT1 (form 1A) having the 1B form (Fig. 5) and strongly suggested the occurrence of intramolecular recombination in the inverted repeats of the pSR1 moiety of the pSRT1 plasmid. Since no DNA sample was a mixture of two isomers, the intramolecular recombination in the inverted repeats must have occurred in the *S. cerevisiae* host, not in the *E. coli* (*recA*) host.

DISCUSSION

Two plasmids, designated pSR1 and pSR2, were discovered in two strains of *S. rouxii*. This

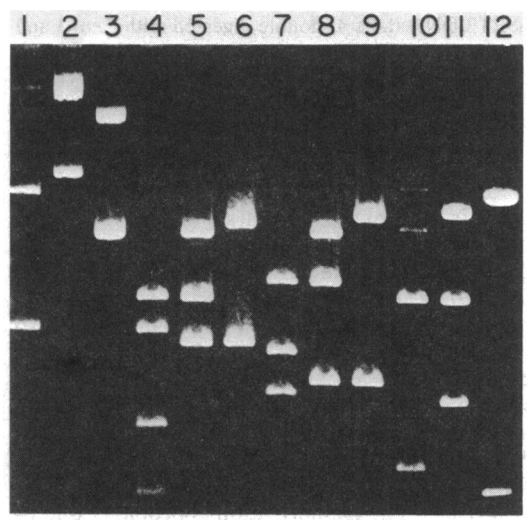

FIG. 7. Restriction analysis of the pSRT1 DNA. pSRT1 DNA digested with the indicated restriction enzyme(s) was run on agarose gel parallel to similarly digested pSR1 or YIp1. Lanes 1 (pSR1), 2 (pSRT1), and 3 (YIp1), undigested DNAs; lanes 4 (pSR1), 5 (pSRT1), and 6 (YIp1), DNAs double digested with *Eco*RI and *Xho*I; lanes 7 (pSR1), 8 (pSRT1), and 9 (YIp1), DNAs double digested with *Eco*RI and *Hin*dIII; lanes 10 (pSR1), 11 (pSRT1), and 12 (YIp1), DNAs digested with *Bam*HI.

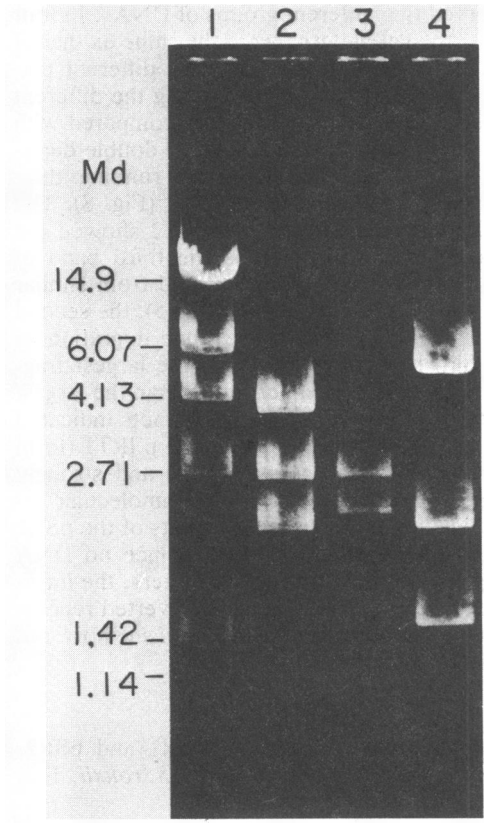

FIG. 8. Restriction analysis of pSRT2 DNA. pSRT2 DNA (lane 4) double digested with *Eco*RI and *Xho*I was analyzed and compared with similarly digested pSRT1 (lane 2) and pSR1 (lane 3) DNAs by agarose gel electrophoresis. Lane 1 is λ DNA digested with *Hin*dIII, and the fragment masses in megadaltons (Md) are indicated in the left-hand margin.

yeast is osmophilic, consists of stable vegetative haploid cells as the dominant phase in the life cycle, and is unable to hybridize with *S. cerevisiae* (29). *S. rouxii* yeast is used in the manufacture of soy sauce and *miso* in Japan and is also responsible for spoilage of foods with high sugar content, such as jam, syrup, and honey (47). Both pSR1 and pSR2 are closed circular DNAs about the same size as the 2-μm DNA plasmid of *S. cerevisiae*. The plasmids exist in two isomeric forms in *S. rouxii*, as does the 2-μm DNA in *S. cerevisiae*. The isomerization is probably due to intramolecular recombination and suggests the presence of a pair of inverted repeats in these plasmids. Although in its gross structure pSR1 resembles 2-μm DNA, the cleavage map of pSR1 differs from that of 2-μm DNA. Furthermore, no sequence homology between pSR1 and 2-μm DNA was detected by Southern hybridization under moderately stringent conditions (65°C in 3× SSC).

DNA plasmids were not found in the majority of the 19 *S. rouxii* strains tested, nor in the 81 strains belonging to nine different species in eight genera that were also examined. This indicates that the occurrence of plasmids in yeasts other than *S. cerevisiae* is rare, and agrees with the finding of Gunge et al. (16), who were able to detect plasmids in one strain of *K. lactis* among 57 strains of yeasts other than *S. cerevisiae* tested. No phenotypic differences were observed due to the presence or absence of the plasmids in *S. rouxii*.

The recombinant plasmid constructed by joining the larger *Xho*I-*Sal*I fragment of YIp1 DNA with pSR1 at the *Xho*I site could replicate in the *S. cerevisiae* host as a plasmid. Since YIp1 does not have an autonomously replicating sequence effective in the *S. cerevisiae* host, pSR1 DNA probably contains a yeast replicon effective in *S. cerevisiae*. Obvious questions that arise are whether the replication origin of pSR1 in *S. rouxii* also initiates the DNA replication of the plasmid in *S. cerevisiae* and what role the putative inverted repeats of pSR1 play. Stinchcomb et al. (39) showed that various DNA fragments from a wide variety of eucaryotic organisms cloned on YIp5 (a chimeric plasmid consisting of pBR322 ligated with a 1.1-kilobase *S. cerevisiae* DNA fragment containing the *URA3* gene but not a replication origin effective in the *S. cerevisiae* host) (41) are capable of autonomous replication in *S. cerevisiae*. Gunge and Sakaguchi (15) have also shown that the linear DNA plasmids associated with the killer character in *K. lactis* are stably maintained and express the killer character in the *S. cerevisiae* host. These facts and the finding that the *S. rouxii* plasmid replicates in *S. cerevisiae* indicate broad similarities in the specificity of the plasmid replication in these eucaryotic organisms and further suggest that 2-μm DNA of *S. cerevisiae* may replicate in *S. rouxii*. To test this, *S. rouxii* strains suitable for use in recombinant DNA experiments in combination with such *S. cerevisiae* vectors as YRp7 and YEp2 (41) are under construction.

Intramolecular recombination of 2-μm DNA occurs within the inverted repeats (2) and may involve one of the proteins (*Able*) encoded by 2-μm DNA (5, 17). The findings that pSR1 has two isomeric forms in *S. rouxii* and that pSRT1, which probably carries the whole molecule of pSR1, except for its *Hin*dIII site, undergoes isomerization in *S. cerevisiae* imply that pSR1 encodes a protein whose function is similar to that of the *Able* protein of 2-μm DNA. However, it remains to be determined whether the isomerization of pSRT1 in the *S. cerevisiae* host depends on its own protein or on the *Able* protein provided by 2-μm DNA, as the strain

D13-1A of *S. cerevisiae* in which the replication of pSRT1 was detected harbors its own 2-μm DNA.

ACKNOWLEDGMENTS

We thank M. Osumi, H. Mori, T. Yamazaki, H. Matsuoka, and C. Shimoda for their generous supply of yeast strains.

This study was done under contract with the Science and Technology Agency of the Japanese Government and was supported by the Special Fund for Multiministerial Projects.

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