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DNA SEQUENCE POLYMORPHISMS IN THE GENUS SACCHAROMYCES. V. CLONING AND CHARACTERIZATION OF A *LEU2* GENE FROM S. CARLSBERGENSIS

by

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Saccharomyces carlsbergensis strains used in the production of lager beer are structurally heterozygous in most genetic loci studied to date.

Previous studies have shown that the genotype of lager yeast contains two types of genomes, one of which is derived from S. cerevisiae and the other reveals similarities to the genomes of S. bayanus and S. monacensis. Genes of homeologous chromosomes can be distinguished by characteristic restriction fragment patterns. This is true also for the *LEU2* genes which encode the β-isopropylmalate dehydrogenase and are located on chromosomes III.

In the present work a *LEU2* gene from S. carlsbergensis has been cloned and characterized. The cloned 2.6 kb *LEU2* region complements the S. cerevisiae *leu2-3 leu2-112* double mutation. The restriction endonuclease site map of the isolated S. carlsbergensis *LEU2* gene is different from that of the S. cerevisiae *LEU2* gene. Electrophoretic chromosome separation, as well as *kar1* mediated transfer of single chromosomes into S. cerevisiae strains, has shown that the S. carlsbergensis specific *LEU2* gene is located on a chromosome III which carries the carlsbergensis specific *HIS4* gene. The cloned *LEU2* gene shows preferential molecular hybridization to one of the two *LEU2* structural alleles present in lager strains, an allele which is also present in type strains of S. bayanus, S. carlsbergensis, S. monacensis and S. uvarum.

1. INTRODUCTION

Previous genetic and molecular studies of chromosomes III (13, 18, 23), V (19), X (7, 8), XII and XIII (25) in lager brewing strains have revealed the presence of at least two homoeologues of each of these chromosomes. These findings have led to the conclusion that the S. carlsbergensis lager yeast is an amphiploid consisting of two or more genomes of which one seems to be derived from a S. cerevisiae strain

Abbreviations: FIGE = field inversion gel electrophoresis; OFAGE = orthogonal field alternation gel electrophoresis; SSC = 150 mM-NaCl, 15 mM-Na citrate; TBE = 89 mM-Tris-borate, 89 mM-boric acid, 2 mM-EDTA.

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while the other shows homology to S. bayanus and S. monacensis strains. Homoeologous genes in the two genomes are distinguishable by their restriction endonuclease fragment patterns. This is also true for the *LEU2* genes, located on chromosomes III, encoding the β-isopropylmalate dehydrogenase (1, 2, 9, 11, 23). The two *LEU2* genes in lager yeast strains can be identified by their SalI diagnostic restriction fragment patterns (23). Pattern III for the S. cerevisiae-like *LEU2* is characterized by the presence of a 16 kb SalI fragment and pattern IV for the S. bayanus and S. monacensis-like *LEU2* by the presence of a 12 kb SalI fragment.

In chromosome III addition and substitution strains the *LEU2* allele, characterized by the SalI fragment IV, co-segregates with the *HIS4* allele displaying the restriction fragment pattern II (13, 18, 23). While the *LEU2* allele associated with SalI fragment III is present in industrial S. carlsbergensis and S. cerevisiae strains (15, 22, 23), the *LEU2* allele associated with SalI fragment IV has, until now, only been detected in S. carlsbergensis lager brewing strains, S. monacensis, S. bayanus and S. uvarum (22, 23, 24).

In the present investigation we describe a 2.6 kb DNA fragment cloned from a genomic library derived of the S. carlsbergensis lager production strain 244. The fragment is (i) able to complement an S. cerevisiae double mutation *leu2-3 leu2-112*, is (ii) located on chromosome III, and (iii) has a restriction map different from that of the S. cerevisiae *LEU2* gene. (iv) The 0.9 kb EcoRI-SalI part of the fragment does not hybridize to the S. cerevisiae *LEU2* sequence under high stringency conditions where it exhibits strong hybridization to similar sequences in type strains of S. bayanus, S. carlsbergensis, S. monacensis and S. uvarum.

2. MATERIALS AND METHODS

2.1. Strains and growth media

The yeast strains used in this study are shown in Table I. As donor for the genomic library the Carlsberg lager strain 244 (syn. BK2246) was used, while the *leu2* double mutant strains of S. cerevisiae IVPX5-2B (26) and DBY746 are the recipient for the selection of the chimeric plasmid containing the *LEU2* region. The other yeast strains shown in Table I have been used for comparative analyses of the cloned *LEU2* insert. Yeast was maintained and grown on complex medium plates (YPD (28)) at room temperature.

E. coli HB101 (recA hsd 5) was used as the host strain in bacterial transformation experiments and for the subsequent preparation of plasmid DNA. The bacteria were grown at 37 °C in LB medium. Ampicillin (50 $\mu g \cdot ml^{-1}$) or tetracycline (15 $\mu g \cdot ml^{-1}$) were added as required for selection of transformants (17).

2.2. Cloning vectors and subcloning of *LEU2*

The cloning vector, kindly provided by M. MCDONELL, was the yeast – E. coli chimeric vector YRp17. YRp17 and the vector pUC13 were used for subcloning of the *LEU2* inserts. Construction of the yeast genomic library from lager strain 244 and transformation of Li^+ treated S. cerevisiae cells by recombinant plasmid DNA preparations were done as previously described (7, 14). Clones which had taken up recombinant plasmids were first isolated on plates of SC minus uracil (28). Upon replica plating to SC minus uracil and leucine (28), clones with plasmids containing a *LEU2* gene were identified by complementation of the *leu2* double mutation.

Plasmid DNA from a yeast transformant demonstrating simultaneous mitotic instability for uracil and leucine independence was isolated, amplified in E. coli HB101 and purified through CsCl centrifugation for subsequent characterization. The plasmid pC 512 (13) contains the SalI-SalI 2.3 kb *LEU2* fragment from S. cerevisiae (29). The 0.9 kb EcoRI-SalI fragment with the down stream end of the S. cerevisiae *LEU2* gene (2) was eluted from a 0.7% (w/v) agarose gel (32) and used as probe or subcloned into the pUC13 vector.

2.3. Preparation of DNA, electrophoretic separation of DNA molecules and hybridization analysis

Plasmid DNA preparations, nick translations, Southern transfers and hybridization conditions for restriction endonuclease analysis were per-

Table I. Characteristics of Saccharomyces strains used in comparative and cloning analyses. *LEU2:* Diagnostic SalI fragment according to (21, 22).

Strain	Genotype/*LEU2*	Source
Bottom fermenting strains:		
S. carlsbergensis:		
Type strain: Bottom fermenting strain No. I		
C84-AJL248	III + IV	Alfred Jørgensen Laboratories
CBS1513	III + IV	Centraalbureau voor Schimmelcultures
Lager strains:		
BK1101 Pschorr lager strain	III + IV	Carlsberg Research Center
BK2208 Danish lager strain	III + IV	Carlsberg Research Center
BK2224 Tuborg lager strain	III + IV	Tuborg
244 syn. BK2246 Carlsberg lager strain	*LEU* donor strain: III + IV	Carlsberg
S. monacensis:		
Type strain: Bottom fermenting strain No. II		
CBS1503	IV	Centraalbureau voor Schimmelcultures
Other Saccharomyces species:		
S. bayanus NCYC374	IV	National Collection of Yeast Culture
S. uvarum CBS395	IV	P. PHILIPPSEN
S. uvarum C81-1511	IV	P. PHILIPPSEN
S. cerevisiae		
S288C	*SUC2 CUP1 gal2*	
DBY746	*leu2-3, leu2-112 MATα his3Δ1 ura 3-52 trp1-289*	
K5-5A	*his4-15 LEU2 MATα ade2-1 can1 kar1-1*	CONDE & FINK (10)
IVPX5-2B	*LEU2* recipient strain: *MATα leu2-3 leu2-112 ura3-52 ilv3-12*	J. POLAINA (26)
Chromosome transfer strains (donor: BK2208, recipient: K5-5A)		
Chromosome III substitution strain:		
CYT-MSP-2208-2	*HIS4 LEU2 MAT***a** *THR4 can1 ade2-1*	PEDERSEN (23)
Chromosome III addition strain:		
CYT-MSP-2208-7	*HIS4 LEU2 MAT***a**/α *THR4 can1 ade2-1*	PEDERSEN (23)
Chromosome transfer strain (donor 244 (syn. BK2246) recipient: K5-5A)		
C80-1253	*HIS4 LEU2 MAT***a** *THR4 can1 ade2-1*	NILSSON-TILLGREN et al. (18)

formed as described earlier (7, 17, 22). Protocols for yeast genomic DNA isolations and full length chromosomal DNA preparations (a modification of the SCHWARTZ and CANTOR protocol (27)) have also previously been described (23).

Electrophoretic separation of the chromosome size DNA molecules was done by orthogonal field alternation gel electrophoresis (OFAGE) (4, 5) and field inversion gel electrophoresis (FIGE) (6). The electrophoresis conditions for FIGE separations were as follows: 1% agarose in 0.5×TBE, 80 Volts at 22 °C. The electrophoresis period was 67 hours with a linear increasing pulse ramp starting at t_{0h} with 9 seconds forward (cathode at side of application) and 3 seconds backwards (anode at side of application). At t_{67h} the forward pulse amounted to 195 seconds and the backwards pulse to 65 seconds.

In order to obtain a binding efficiency with sulfonated probes, which is comparable to that of ^{32}P-labelled probes, hybridizations of Southern blots after FIGE separations were done in higher salt solutions (6×SSC) than previously used with gels after OFAGE (3×SSC) (23). Washes for low stringency conditions with sulfonated hybridizations probes were in 6×SSC, 0.2% SDS at 58 °C, medium stringency filter washes were done in 3×SSC, 0.2% SDS at 58 °C, while high stringency filter washes were done in 1×SSC, 0.2% SDS at 58 °C. Autoradiographic exposures ranged from 12 hours to 3 days at -80 °C using Kodak X-Omatic intensifying screens on Kodak XAR5 X-ray films, while the non-radioactive detection of sulfonated probes on nitrocellulose membranes was made with the CHEMIPROBE (FMC-LITEX) and SULFOPROBE (SIGMA) systems. In both systems the cytosine groups are sulfonated by the method of SVERDLOV et al. (30). The modified cytosines are recognized by the primary antibody, which is subsequently made visible with a secondary antibody alkaline phosphatase complex. As suggested by LEBACQ et al. (16) and the manual of the CHEMIPROBE system, the sulfonated probes were used in high concentrations, i.e. in the range of 0.5-2 $\mu g \cdot ml^{-1}$ and in a total volume of 20-25 ml in a plastic bag. Radioactively labelled probes were used at a concentration in the range of 4-10 $ng \cdot ml^{-1}$ in a total volume of 100-200 ml in a plastic tray. Antibody concentrations and blocking solutions were used as prescribed by the CHEMIPROBE protocol, while all washings of filters after the primary and secondary antibody reactions were done in 0.5 M-NaCl, 0.3% Brij 35 (16).

2.4. *LEU2* probes

Four different *LEU2* probes were used during the course of this study: (i) the 0.9 kb EcoRI-SalI 3' fragment of *LEU2* from the S. cerevisiae SalI-SalI insert in the vector pC512 (13). (ii) The pKh1-1 plasmid containing the original Sau3AI generated *LEU*$^+$ insert from the S. carlsbergensis genomic library, (iii) the 2.8 kb SalI-SalI *LEU2* containing fragment from the plasmid pKh2-1 (cf. Figure 1), (iv) the 0.9 kb EcoRI-SalI fragment from pKh2-1 after subcloning in pUC13.

3. RESULTS

3.1. Restriction endonuclease site analysis of the cloned *LEU2* allele

Saccharomyces cerevisiae IVPX5-2B (*leu2-3, leu2-112*) was transformed with 10 μg of recombinant plasmid DNA from each of four pools of the S. carlsbergensis genomic library as described by CASEY (7). Of the 3000-4000 Ura$^+$ transformants arising from each of the pools on plates of SC minus uracil, one clone was able to grow on plates of SC minus uracil minus leucine. After verification that the Ura$^+$Leu$^+$ phenotype was unstable when grown on SC medium, the plasmid designated pKh1-1 was analysed by restriction endonuclease mapping.

Identification of the *LEU2* containing sequences with in the 5.65 kb insert in pKh1-1 was done by hybridization of the 0.9 kb EcoRI-SalI *LEU2* fragment from pC512 to a Southern blot of EcoRI digested, SalI digested and EcoRI + SalI digested plasmid. Three fragments from pKh1-1 hybridized to the EcoRI-SalI probe, namely: a 1.2 kb EcoRI fragment, a 2.8 kb SalI fragment and an 0.9 kb EcoRI-SalI fragment. Subsequently, it was found that the 2.8 kb SalI-SalI fragment in this plasmid, when subcloned in YRp17, retained the ability to complement the *leu2* mutations in S. cerevisiae IVPX5-2B and DBY746. This plasmid, designated pKh2-1, contains an insert of 2.6 kb. If DBY746

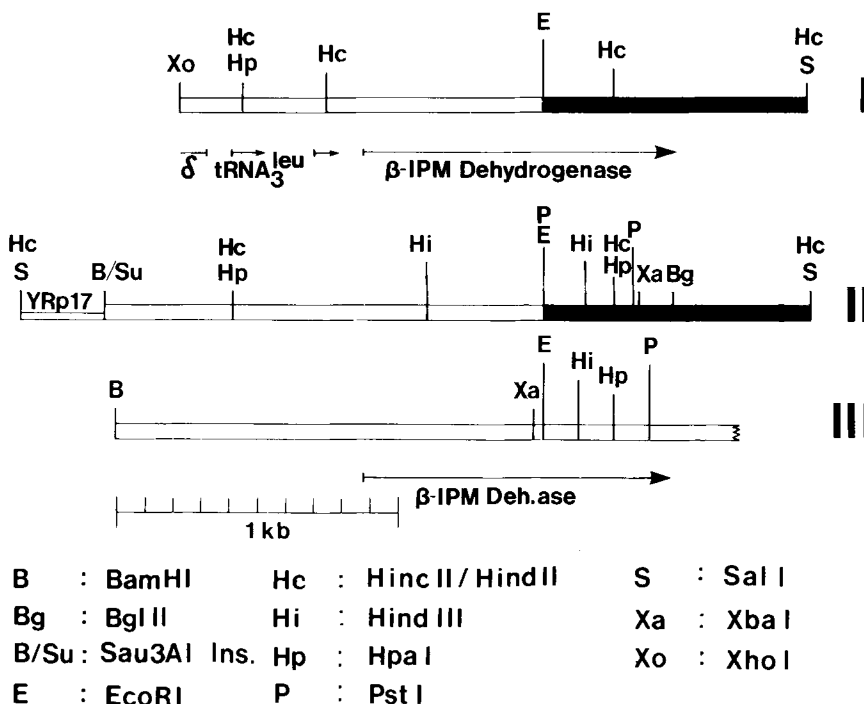

Figure 1. Restriction site maps of three *LEU2* genes. I) XhoI-SalI *LEU2* restriction endonuclease fragment from S. cerevisiae (1, 2), II) SalI-SalI *LEU2* fragment from lager strain 244. III) Part of BamHI-XhoI Candida maltosa *LEU2* fragment (31). The restriction endonuclease sites in I) and III) are based on the published sequences. Filled areas in I) and II) correspond to the subcloned EcoRI-SalI fragments, respectively, designated as the 0.9 kb EcoRI-SalI fragments from S. cerevisiae and S. carlsbergensis.

transformed with pKh2-1 is plated on SC minus uracil (non-selective conditions for leucine) instead of SC minus leucine or SC minus leucine minus uracil, several *URA*$^+$*LEU*$^-$ colonies are found among the transformants. The instability for the *LEU*$^+$ phenotype is presumably due to the duplication of the BamHI-SalI region of the E. coli tetracycline gene in pKh2-1. The duplication might induce a cross-over event which loops out the *LEU2* gene. When the 2.6 kb insert was hybridized to Southern blots of digests of genomic DNA preparations from S. carlsbergensis 244 and S. cerevisiae S288C, cross hybridization to a variety of fragments was seen in both strains, indicating the presence of repeated DNA sequences. Such repeated sequences have been found upstream of the S. cerevisiae *LEU2* gene (tRNA and δ-sequences (1, 2, 11)). To avoid cross hybridization of unwanted sequences, the 0.9 kb EcoRI-SalI fragment from pKh2-1 was chosen as probe.

The SalI-SalI fragment was mapped for restriction sites with the endonucleases listed in Figure 1. The resulting map of the cloned carlsbergensis *LEU2* gene reveals six restrictions sites in the coding region which are absent in the sequenced S. cerevisiae *LEU2* gene (1, 2). Additional restriction site polymorphisms are found in the adjacent region.

3.2. Identification of the cloned *LEU2* allele

Genomic DNA from the Carlsberg lager strain 244 and S. cerevisiae (S288C) were cut with seven restriction endonucleases and the fragments separated on two gels under identical conditions and blotted onto filters. One filter was hybridized first at non-stringent conditions

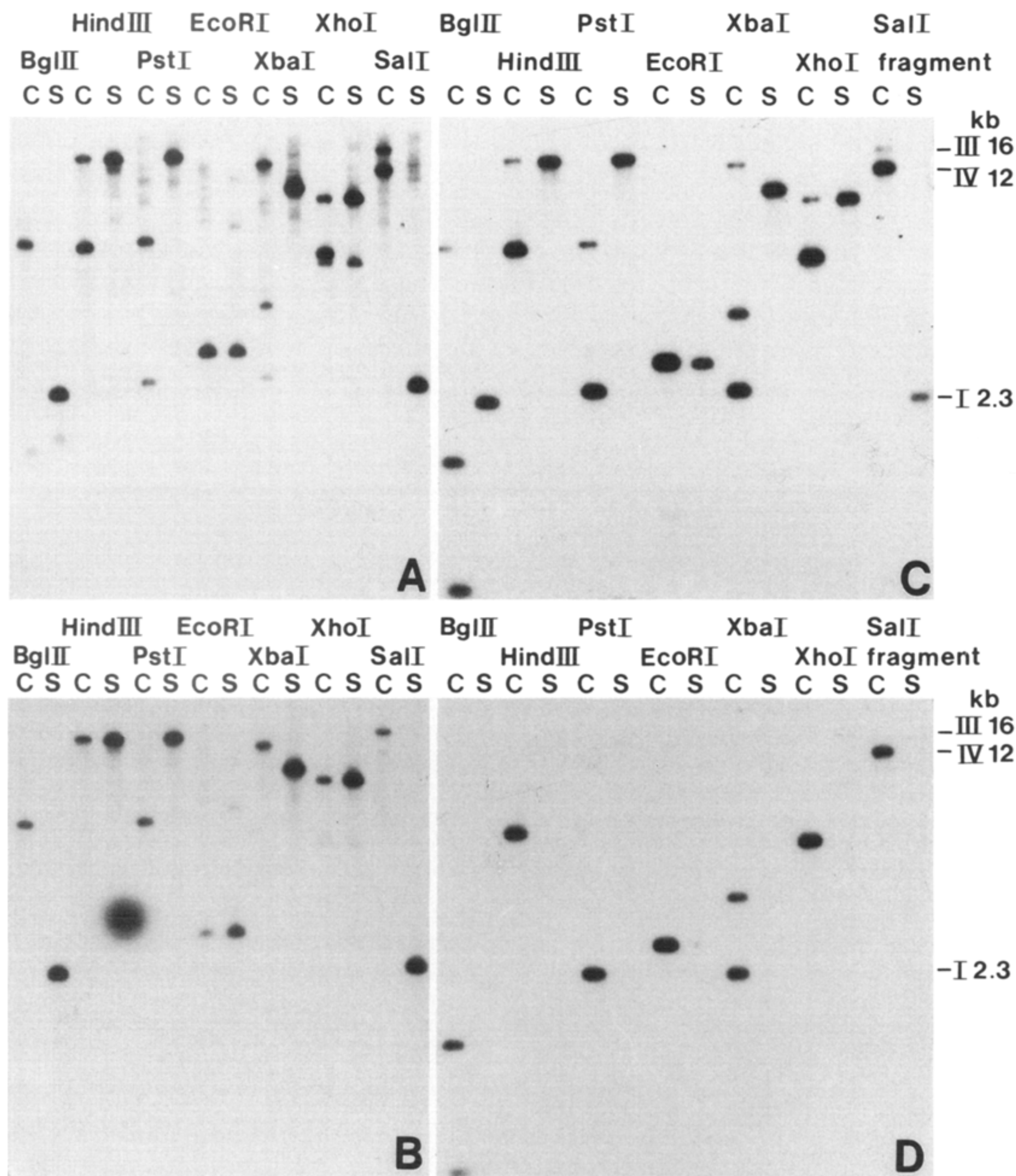

Figure 2. Molecular hybridization of endonuclease digested DNA from S. cerevisiae and lager strain 244 with the 0.9 kb ^{32}P-labelled EcoRI-SalI *LEU2* probes from S. cerevisiae S288C and lager strain 244. Panels A and B are from one filter which has been successively hybridized with the 0.9kb EcoRI-SalI *LEU2* fragment from plasmid pC512 with an insert of S. cerevisiae at low and high stringency, while panels C and D are from a filter which has been hybridized successively with the 0.9 kb EcoRI-SalI *LEU2* fragment from plasmid pKh2-1 with an insert of the lager strain 244 at low and high stringency. Filters in panels A and C have been hybridized and washed at 60 °C and 3×SSC, while the same two filters shown in panels B and D have been washed subsequently at high stringency, namely 68 °C and 0.1×SSC. Lanes containing DNA from lager strain 244 are marked with a C while lanes containing DNA from S. cerevisiae S288C are marked with an S.

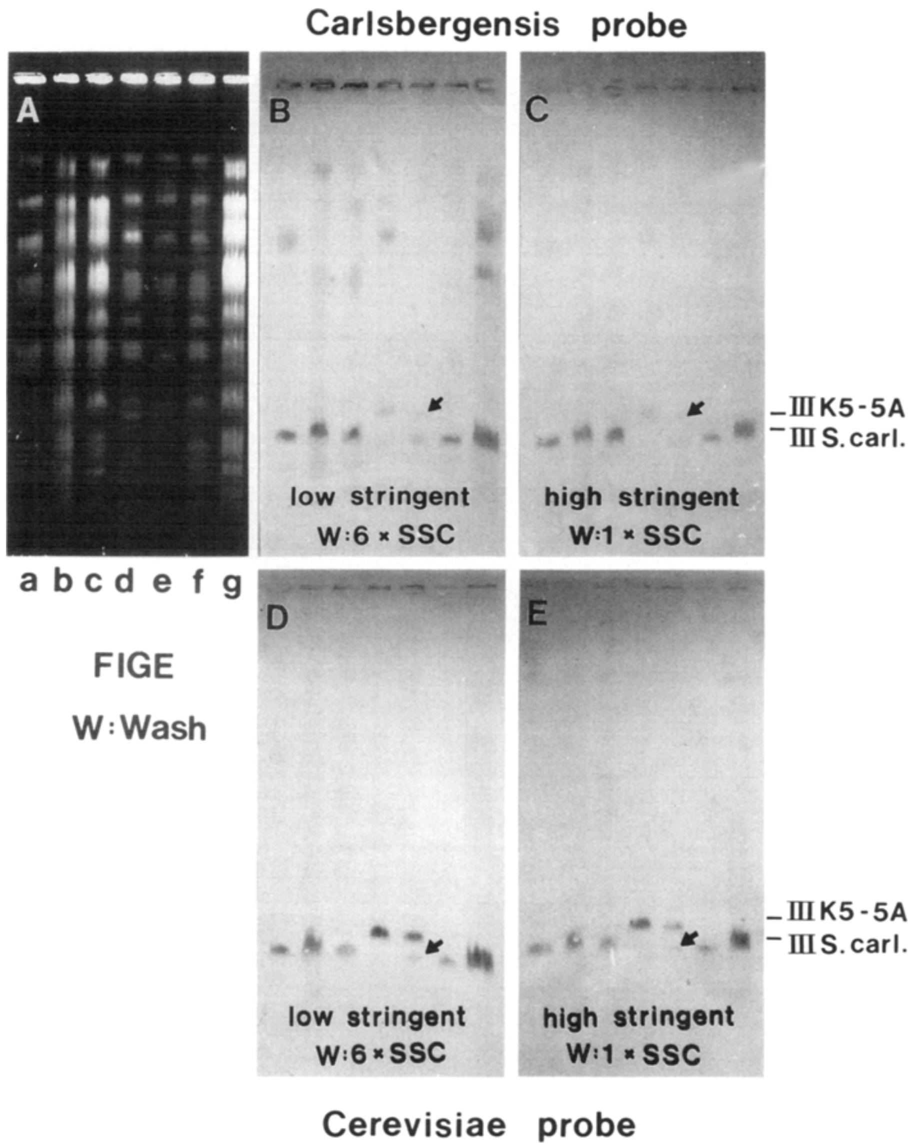

Figure 3. Panel A: FIGE separation of chromosomal DNA preparations from seven Saccharomyces strains. a: C80-1253 a chromosome III substitutionstrain, b: Carlsberg lager strain 244 (BK2246) donor of chromosome III in C80-1253, c: Pschorr lager BK 1101, d: K5 – 5A chromosome III receptor strain, e: CYT-MSP-2208-7, chromosome III addition strain with a chromosome III from BK2208, f: CYT-MSP-2208-2, chromosome III substitution strain with BK2208 as chromosome III donor, g: BK2208 Danish lager strain. Panels B to E: Four filters prepared from four electrophoretic separations as that depitcted in A. The filters were hybridized to *LEU2* probes in 6×SSC at 58 °C. Panels B and D: Filters were washed in 6×SSC at 58 °C. Panels C and E: Filters washed in 1×SSC at 58 °C. Filters in panels B and C have been hybridized with the sulfonated 0.9 kb EcoRI-SalI S. carlsbergensis *LEU2* probe, while the filters depicted in panels D and E were hybridized with the corresponding sulfonated S. cerevisiae probe. Panels B to E: Black arrows point at weakly hybridizing bands.

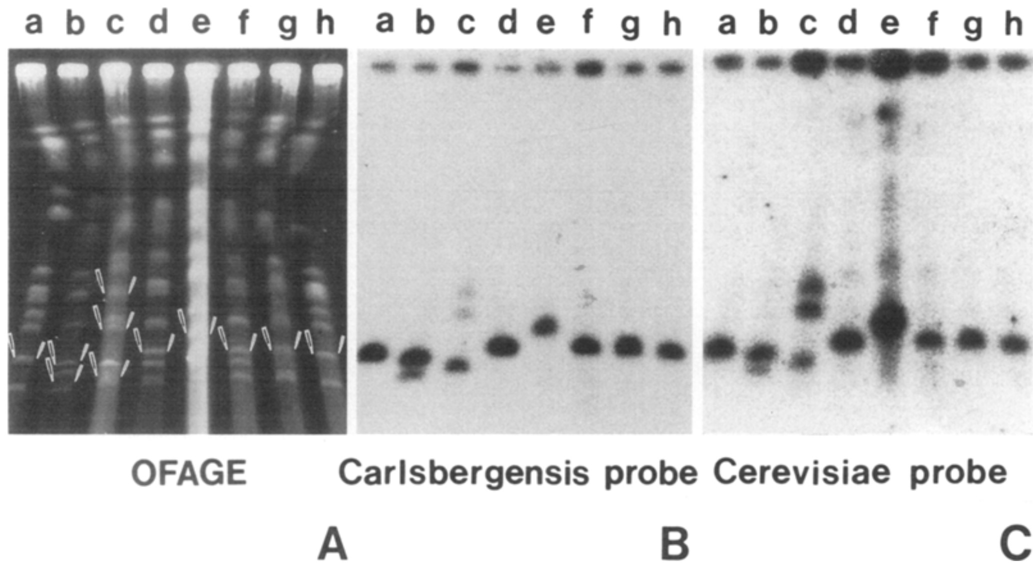

Figure 4. Panel A: OFAGE separations of chromosomal DNA preparations from 8 Saccharomyces strains. Lane a: S. uvarum CBS395, lane b: S. monacensis CBS1503, lane c: S. carlsbergensis C84-AJL248, lane d: S. carlsbergensis Carlsberg lager strain 244 (syn. BK2246), lane e: S. cerevisiae laboratory strain S288C, lane f: S. carlsbergensis Pschorr lager strain BK1101, lane g: S. bayanus NCYC374 and lane h: S. uvarum C81-1511. Black arrow heads on the left hand side of lanes correspond to *HIS4* hybridizing bands (23) and white arrow heads on the right hand side of the lanes correspond to the *LEU2* hybridizing bands visualized in panels B and C. Panels B and C: Two hybridizations of the Southern blot from the gel in panel A. The chromosomal DNA blot has been hybridized with either the ^{32}P-labelled 0.9 kb EcoRI-SalI *LEU2* fragment from Carlsberg lager strain 244 (panel B) or with the 0.9 kb EcoRI-SalI *LEU2* fragment from S. cerevisiae (panel C). The filter has been hybridized and later washed at 60 °C and 3×SSC in both cases. The lettering of the strains corresponds to panel A.

and subsequently at stringent conditions to the 0.9 kb ^{32}P-labelled EcoRI-SalI 3' *LEU2* fragment of S. cerevisiae (Figure 2A and B). The filter of the other gel was hybridized in the same way to the corresponding *LEU2* fragment from S. carlsbergensis (Figure 2C and D). The comparison reveals that the lager strain 244 contains at least two *LEU2* genes: One *LEU2* allele which is diagnosed with the aid of SalI fragment III gives with HindIII, EcoRI and XhoI digestions the same restriction fragment patterns as the *LEU2* allele from S. cerevisiae S288C (Figure 2B). The other *LEU2* allele which is diagnosed with the aid of SalI fragment IV gives under stringent conditions with the carlsbergensis probe rise to unique restriction fragment patterns with the endonucleases tested (Figure 2D).

In Figures 2A and 2C are compared autoradiographs of the filters hybridized with the S. cerevisiae 0.9 kb EcoRI-SalI *LEU2* fragment from the vector pC512 (Figure 2A) and the 0.9 kb EcoRI-SalI *LEU2* fragment cloned from the Carlsberg lager strain and subcloned into pUC13 (Figure 2C). The hybridization and washing conditions for low stringency were both times 3×SSC at 60 °C. The two autoradiographs yielded the same restriction fragment patterns but fragments hybridising weakly with the cerevisiae derived probe hybridize strongly with other and vice versa. Complementary restriction fragment patterns are obtained at high stringency hybridization conditions (Figures 2B and 2D).

In previous genetic and molecular studies it was found that the carlsbergensis specific *LEU2* and *HIS4* alleles were linked and located on a chromosome III which in orthogonal field alternation gel electrophoresis (OFAGE) moves

faster than the corresponding chromosome III from S. cerevisiae (23, 24). To demonstrate that the cloned *LEU2* gene from S. carlsbergensis originates from the faster migrating (shorter) chromosome III, the 0.9 kb EcoRI-SalI fragments from S. cerevisiae and S. carlsbergensis *LEU2* were hybridized to Southern blots of chromosome III addition and substitution strains. The ethidium bromide stained gel depicted in Figure 3A contains chromosomal DNA from seven Saccharomyces strains separated by the FIGE system. Three additional gels with the same DNA samples have been prepared and all four were then blotted on filters. Hybridizations with the two *LEU2* probes were done under stringent (Figure 3C and E) and less stringent conditions (Figure 3B and D). The detection of *LEU2* containing chromosomal bands has been done by antibody recognition of sulfonated probes. Filters in Figure 3, panels B and C were hybridized with pUC13 containing the S. carlsbergensis 0.9 kb EcoRI-SalI insert, while the filters depicted in panels D and E were hybridized with the corresponding S. cerevisiae probe.

At low stringency washes both probes detect the chromosomes III in all strains, but with preferential hybridization to chromosomes carrying similar or identical *LEU2* alleles. This differential effect is pronounced in the chromosome III addition strain CYT-MSP-2208-7 (strain e), where the S. carlsbergensis *LEU2* probe shows stronger binding to the faster moving chromosome III (panel B), while the S. cerevisiae *LEU2* probe binds preferentially to the slower moving chromosome III originating from K5-5A (panel D). At high stringency washes the preferential hybridization of the probes is similar (panels C and E). To confirm that the cloned *LEU2* gene is located on similarly sized chromosomes III in other Saccharomyces species the 0.9 kb EcoRI-SalI fragments subcloned from pC512 and pKh2-1 were hybridized to the Southern blot of the OFAGE gel shown in Figure 4. In panel A, bands hybridizing to the *HIS4* probe (pC503 (12)) are indicated by black arrow heads (left side of lanes) and *LEU2* hybridizing bands indicated by white arrow heads (right side of lanes). In all strains the *HIS4* and *LEU2* hybridizing bands coincide and thus the bands contain chromosome III or at least the part of chromosome III which corresponds to the left arm of chromosome III in S. cerevisiae. It should be mentioned that S. monacensis CBS1503 contains two chromosomal bands hybridizing with *LEU2* and S. carlsbergensis C84-AJL248 contains three. The slower chromosomal band hybridizing with *LEU2* in S. monacensis has a size and hybridization intensity similar to such a chromosome in the lager strains, while the shorter chromosomal band is of a size and a hybridization intensity similar to the shortest of the three with *LEU2* hybridizing chromosomal bands in S. carlsbergensis C84-AJL248.

4. DISCUSSION

The present investigation has shown the existence of one functional *LEU2* gene in S. carlsbergensis, which contains unique restriction sites compared to the corresponding gene from S. cerevisiae.

The cloned fragment exhibits restriction site similarities to the *LEU2* gene from Candida maltosa (31) (Figure 1), even though C. maltosa seems not closely related to the S. cerevisiae group. The Mol% of G+C of nuclear DNA from C. maltosa is in the range 35.6-37.3 while the Mol% G+C of the S. cerevisiae group is in the range of 38.8-42.0 (3).

Chromosomal diversity is observed among the strains of Saccharomyces when probed with sequences from the two *LEU2* alleles. For example, while the Pschorr lager strain is homozygous for the *HIS4* pattern II allele (20) and heterozygous for the two *LEU2* alleles (22), the S. monacensis strain is homozygous for *HIS4* pattern II as well as the carlsbergensis specific *LEU2* allele with the diagnostic SalI fragment IV (22). Of potentially evolutionary significance is the observation that S. bayanus and S. monacensis yeasts possess a chromosome III with the same size and the carlsbergensis specific *LEU2* allele, as found in Carlsberg lager strain 244.

The most distinct diversity in size of chromosome III was found in type strains of S. carlsbergensis (e.g. C84-AJL248). The strain was found to be trisomic for chromosome III (or at least containing three chromosomes III left arms)

with all three chromosomes migrating differently of chromosome III from other lager strains. The two longer chromosomes III contained both a S. cerevisiae type of *LEU2* (judged by the preferential hybridization), while the short chromosome contained the carlsbergensis specific *LEU2* allele. It has previously been found (23) that the two longer chromosomes also show preferential hybridization to *HIS4* from S. cerevisiae (cf. also Figure 4).

Interestingly, the S. monacensis type strain CBS1503 might well be trisomic for chromosome III, containing only the carlsbergensis specific alleles for *LEU2* and *HIS4*. Both chromosomal bands show preferential hybridization to the carlsbergensis specific alleles and the slower migrating band hybridizes with an intensity twice as strong as the faster moving band (Figure 4). This indicates that the slower moving band contains two copies of chromosome III.

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