MOLECULAR AND GENETIC ANALYSIS OF CHROMOSOMES X IN SACCHAROMYCES CARLSBERGENSIS

by

GREGORY PAUL CASEY

Department of Physiology, Carlsberg Laboratory
Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby

Present address: Department of Applied Microbiology and Food Science,
University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0, Canada

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Molecular hybridization of electrophoretically separated and blotted chromosomes with *ILV3* gene probes detected the presence of three chromosomes X in *S. carlsbergensis* 244 lager yeast. Meiotic segregants, which were disomic or trisomic for chromosome X were crossed with a *kar1-1* strain of *S. cerevisiae* (*MAT α* or *MAT α arg3 met3 ilv3 cycl-1 cdc11 hom6 ade2-40 cyh2 kar1-1*) for single chromosome transfer and isolation of two of the chromosomes X from the lager yeast. Type I chromosome X migrates slower than a reference chromosome X of *S. cerevisiae* K5-5A during chromosome separation by electrophoresis and contains the allele of *ILV3* unique to the lager yeast. It recombines with chromosome X from *S. cerevisiae*, but only in the left arm. Type II chromosome X migrates faster than the reference chromosome X and contains the allele of *ILV3* also found in *S. cerevisiae* S288C. It recombines only in the right arm with chromosome X from *S. cerevisiae*. The third chromosome X from *S. carlsbergensis*, which co-migrates with the reference chromosome X of *S. cerevisiae* K5-5A, has yet to be isolated into the genetic background of a genetic standard strain.

1. INTRODUCTION

In *Saccharomyces cerevisiae*, each of the structural genes for the first four steps of the isoleucine-valine pathway have been mapped and cloned (8, 25, 26, 27). Unfortunately, knowledge obtained in studies with *S. cerevisiae* yeast cannot be directly applied in experiments attempting to genetically analyze and/or engineer lager yeast strains as these yeasts generally exhibit no or poor mating ability, poor sporula-

tion and low spore viability (1, 9, 14, 32, 35). Attempts to overcome these difficulties have been limited by a lack of detailed knowledge concerning the genetic composition of *S. carlsbergensis* lager yeast. There is, however, a general belief in the brewing industry that lager yeasts are polyploid (7, 16, 28, 32) and that this property, besides interfering with mating ability, causes irregularities in meiosis that result in poor sporulation and low spore viability.

Abbreviations: EDTA = ethylene diamine tetracetic acid, sodium salt; kb = kilo base; NPD = non parental ditype; PD = parental ditype; SC = synthetic complete medium; SD = synthetic dextrose medium; SDS = sodium dodecyl sulphate; SSC = 0.15 M-NaCl, 15 mM-Na citrate, pH 7.0; TE = 10 mM-Tris · HCl, 1 mM-EDTA, pH 7.5; Tris = tris-(hydroxymethyl)-amino methane; TT = tetra type; YPD = yeast extract peptone dextrose medium.

In recent years, however, the technique of single chromosome transfer (6, 21) has made it possible to isolate and analyse individual wild-type chromosomes from a *S. carlsbergensis* lager yeast in the genetic background of a *S. cerevisiae* standard strain. The technique is based on the *kar1* mutation which dramatically reduces the frequency of karyogamy after zygote formation (5) but still permits chromosome transfer between nuclei. Thus in *kar1* × *KAR1* crosses one or several chromosomes can be transferred between nuclei during the transient heterokaryotic state (6, 19, 21).

In this manner chromosomes III, V, XII and XIII (with *ILV1*, *ILV5* and *ILV2* located on the latter three chromosomes, respectively) have been isolated and studied from a *S. carlsbergensis* lager yeast (19, 21, 34). Genetic recombination and molecular hybridization experiments have been very revealing as it is now apparent that these chromosomes exist in the lager yeast in two copies. In general, one of the chromosomes is closely homologous to the same chromosome from *S. cerevisiae* and can recombine extensively with it (19, 21, 34). The second chromosome, called the homeologous chromosome, contains the same genes in the same order as those found in the *S. cerevisiae* and homologous *S. carlsbergensis* chromosomes, but it is largely unable to recombine with its counterpart *S. cerevisiae* chromosome. Molecular hybridization studies have shown that genes on the homeologous chromosomes have a different structure at the nucleotide sequence level than genes in the homologous chromosomes (12, 20). Direct evidence for this has been obtained in the case of *ILV1* where analysis of the nucleotide sequence in a 276 base long region of the open reading frame in the *ILV1* allele of *S. cerevisiae* and the homeologous *ILV1* allele in *S. carlsbergensis* has revealed only 84% sequence homology (C. GJERMANSEN, personal communication). Chromosome X contains the *ILV3* gene, encoding for dihydroxyacid dehydrase, and two alleles of this gene have recently been cloned and analyzed by CASEY (4) from *S. carlsbergensis* 244. The presence of two alleles suggests that chromosome X also exists in this yeast as a homologous/homeologous pair.

The production of vicinal diketone aroma

compounds during wort fermentation, especially diacetyl and pentanedione, is of considerable importance to the brewing industry as consumers prefer low levels of these in beer (2). Diacetyl and pentanedione are not actually produced by brewers' yeast but are side-products of the isoleucine-valine pathway. Excess acetohydroxy acids formed by acetohydroxy acid synthase are excreted during the primary fermentation into the wort. A non-enzymatic oxidative decarboxylation follows with acetolactate being converted to diacetyl and acetohydroxybutyrate to pentanedione. A major reason for costly secondary fermentation in brewing is to reduce the levels of vicinal diketones during storage. A genetically engineered brewing yeast which produces low levels of these compounds would therefore be of great value to the brewing industry (34). In this publication, as part of an overall program at the Carlsberg Laboratory to genetically engineer lager yeast strains producing low levels of diacetyl, the isolation and analysis of two of three chromosomes X from *S. carlsbergensis* 244 lager yeast is reported.

2. MATERIALS AND METHODS

2.1. Strains and media

All media used in this study have been described previously (15, 26). The strains of *S. cerevisiae* and *S. carlsbergensis* employed throughout this study are listed in Table I. Figure 1 illustrates the crosses performed to construct the C85-0605 recipient and tester strains used in the isolation and analysis of chromosomes X. *S. cerevisiae* K5-5A was the source of the *kar1-1* mutation and of the 7 chromosome X markers in the starting strains all but *ura2* was in the final C85-0605 strains. The *ura2* marker was lost in the C84-0210 × C84-1010 crosses (Figure 1) as all C84-0411 progeny scored as wild-type *URA2*. Only later in the course of this study was it discovered that a psi factor was involved with this phenomenon as many C84-0411 clones originally scored as *URA2* could be scored as *ura2* after 2 cycles of growth in the presence of 5 mM-guanidinium chloride. C84-0411-A64 and C84-0411-A33 were both subsequently found to be *URA2*. The linkage map for the six

G.P. CASEY: Chromosome X of carlsbergensis

Table I. Yeast strains used in the study of chromosome X

S. carlsbergensis strains		
244	Production lager brewing strain of Carlsberg Breweries	
C80-CG7	Spore derived clone of 244, MATa	(10)
C80-CG65	Spore derived clone of 244, MATa	(10)
C80-CG110	Spore derived clone of 244, MATa	(10)
S. cerevisiae strains used to construct tester and recipient C85-0605 strains		
K5-5A	MATa his4-594 ade2-10 can1 kar1-1	(5)
M607	MATa his4-594 ade2-40 trp5 lys1 cyh2	Carlsberg Laboratory
C82-0712-A30	MATa ura2 adel rad1	T. Nilsson-Tillgren
C83-701-C8	MATa ura2 adel can1 kar1-1	"
C83-0701-C36	MATa ura2 adel can1 kar1-1	"
C84-3004-B23	MATa met3 ilv3 cyo1-1 odc11 hom6	"
C84-3004-B35	MATa met3 ilv3 cyo1-1 odc11 hom6	"
C84-0108-17	MATa leu2-1 arg3	"
C84-0210-A1	MATa ura2 ade2-40 trp5 lys1 cyh2 kar1-1	This study
C84-0210-B4	MATa ura2 ade2-40 lys1 cyh2 kar1-1	"
C84-1010-A35	MATa arg3 cyo1-1 odc11 hom6	"
C84-0411-A33	MATa arg3 cyo1-1 ade2-40 trp5 lys1 cyh2 kar1-1	"
C84-0411-A64	MATa arg3 cyo1-1 hom6 ade2-40 lys1 cyh2 kar1-1	"
C84-2812-A2	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 adel trp5 cyh2	"
C84-2812-A9	MATa arg3 ade2-40 cyh2	"
C84-2812-A44	MATa met3 ilv3 cyo1-1 odc11 hom6	"
C84-2812-A61	MATa arg3 hom6 ade2-40 lys1 cyh2 kar1-1	"
S. cerevisiae C85-0605 recipient strains for chromosome X transfer		
C85-0605-A5	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 ade2-40 cyh2 kar1-1	"
C85-0605-A10	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 ade2-40 lys1 cyh2 kar1-1	"
C85-0605-A24	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 ade2-40 cyh2 kar1-1	"
C85-0605-B3	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 ade2-40 lys1 cyh2 kar1-1	"
C85-0605-B21	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 ade2-40 cyh2 kar1-1	"
C85-0605-B40	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 ade2-40 lys1 cyh2 kar1-1	"
S. cerevisiae C85-0605 tester strains for chromosome X analysis		
C85-0605-B2	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 KAR1	"
C85-0605-A33	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 KAR1	"
C85-0605-B10	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 lys1 cyh2 KAR1	"
C85-0605-B19	MATa arg3 met3 ilv3 cyo1-1 odc11 hom6 KAR1	"
S. cerevisiae strains containing a S. carlsbergensis chromosome X subjected to tetrad analysis		
C85-0605-A16	C85-0605-A5 with an additional chromosome X from C80-CG7	"
C85-0605-A29	C85-0605-A5 with chromosome X substituted with a chromosome X from C80-CG7	"
C85-0605-C2	C85-0605-B40 with chromosome X substituted with a chromosome X from C80-CG7	"
C85-0605-M31	C85-0605-A10 with an additional chromosome X from C80-CG65	"
S. cerevisiae strains containing a S. carlsbergensis chromosome X which could not complement cyo1-1 and for which there is no tetrad analysis data		
C85-0605-A78	C85-0605-A5 containing a S. carlsbergensis chromosome X from C80-CG7	"
C85-0605-A148	"	"
C85-0605-B6	C85-0605-A10 containing a S. carlsbergensis chromosome X from C80-CG7	"
C85-0605-B18	"	"
C85-0605-C48	C85-0605-B40 containing a S. carlsbergensis chromosome X from C80-CG7	"
C85-0605-C63	"	"
C85-0605-C85	"	"
C85-0605-C87	"	"
C85-0605-K12	C85-0605-B21 containing a S. carlsbergensis chromosome X from C80-CG110	"
C85-0605-L28	C85-0605-A5 containing a S. carlsbergensis chromosome X from C80-CG65	"
C85-0605-L98	"	"
C85-0605-L100	"	"
C85-0605-M13	C85-0605-A10 containing a S. carlsbergensis chromosome X from C80-CG65	"
C85-0605-M27	"	"
C85-0605-M36	"	"
C85-0605-M44	"	"
C85-0605-N14	C85-0605-A24 containing a S. carlsbergensis chromosome X from C80-CG65	"
C85-0605-N20	"	"
S. cerevisiae strains containing a S. carlsbergensis chromosome X which can complement cyo1-1 and for which there is no tetrad analysis data		
C85-0605-A1	C85-0605-A5 containing a S. carlsbergensis chromosome X from C80-CG7	"
C85-0605-A2	"	"
C85-0605-A6	"	"
C85-0605-A7	"	"
C85-0605-A11	"	"
C85-0605-A19	"	"
C85-0605-A24	"	"
C85-0605-A27	"	"
C85-0605-A28	"	"
C85-0605-A33	"	"
C85-0605-A38	"	"
C85-0605-A39	"	"

Figure 1. Crosses employed in the construction of *S. cerevisiae* C85-0605 recipient and tester strains.

markers in chromosome X of *S. cerevisiae* is shown in Figure 2.

Map distances between the *arg3* to *cdc11* chromosome X markers in the C85-0605 strains (Table II) were estimated by the formula of PERKINS (24) and are in good agreement with those published in the Edition 9 genetic map of *Saccharomyces cerevisiae* (18). In a separate cross between C84-2812-A2 (*MATa ilv3 hom6 arg3 met3 cye1-1 cdc11 trp5 ade1 cyh2*) and C84-2812-A9 (*MATa ade2-40 arg3 cyh2*) the distance between the *cdc11* and *hom6* loci was estimated at 43.8 cM (PD:2, NPD:0, TT:14), compared to the reported distance of 37.5 cM (18).

2.2. Chromosome transfer protocol

The mass mating technique used for chromosome transfer was based on the protocol of

JAKOB (13). Two strains of different mating type were grown to late log phase on YPD plates. Two loopfuls of each strain were then scraped off the surface of the plates and mixed with each other on a fresh YPD plate (several loops of sterile water were used to ensure homogenous mixing). The mass mating mixture was incubated at 30 °C for 4-6 hours followed by overnight incubation at 20 °C. The cells were then rinsed off the plate, washed twice with sterile water, and plated at a high density onto plates of selective media.

2.3. Tetrad analysis and scoring of chromosome X markers

Standard techniques were used for sporulation and tetrad analysis (11). The markers *arg3*, *met3*, *ilv3* and *hom6* were scored as the inability to grow at 30 °C on plates of SC-arginine,

Figure 2. Map of relevant markers of chromosome X in *S. cerevisiae* (18) and of type I and type II chromosomes X in *S. carlsbergensis*. The regions in chromosome X of *S. carlsbergensis* which recombine with the reference chromosome X of *S. cerevisiae* are indicated by crosses.

SC-methionine, SC-isoleucine-valine and SC-threonine, respectively (while being able to grow on SC medium). The *cdc11* marker was scored as the inability to grow at 37 °C on YPD plates (but being able to grow at 30 °C on YPD). The *cyc1-1* marker is a deletion (31) covering the closely linked genes *CYC1 OSM1 RAD7* and was scored for the *rad7* phenotype i.e. as the inability to grow on plates of YPD agar after 5 seconds exposure to UV light and incubation in the dark at 30 °C.

2.4. Molecular hybridization analysis

Extraction, cleavage, electrophoretic separation and transfer of DNA onto nitrocellulose paper were carried out following the protocol of MANIATIS et al. (17). Nick translation procedures and hybridization conditions were as described earlier (4). Filters containing hybridized probes were washed under conditions of high stringency (i.e. 68 °C, 0.1×SSC) or low stringency (i.e. 60 °C, 3×SSC) depending on the nature of the probe and the experimental objective.

Table II. Tetrad analysis of the cross C84-2812-A61 (*MAT α ade2-40 lys1 hom6 arg3 cyh2 kar1-1*) X C84-2812-A44 (*MAT α ilv3 cdc11 hom6 met3 cyc1-1*)

	PD	NPD	TT	Calculated Distances (cM)	Reported Distances (cM)*
<i>arg3-met3</i>	7	11	32	98	60.6
<i>met3-ilv3</i>	38	0	7	7.8	7.3
<i>ilv3-cyc1-1</i>	31	0	40	28.4	24.7
<i>cyc1-1-cdc11</i>	43	0	29	20.1	20.8

* As reported in the genetic map, edition 9, of *Saccharomyces cerevisiae* (18)

BglII- SalI fragment hybridizing	Pattern I DNA: <i>S. cerevisiae</i> probe: (i) 16-1 (high stringency)	Pattern I + II DNA: <i>S. carlsbergensis</i> probe: (i) 16-1 probe: (ii) 12-1 (low stringency)	Pattern II DNA: <i>S. carlsbergensis</i> probe: (ii) 12-1 (high stringency)
6.8 kb	+	+	
3.9kb 3.9kb	+	+	+
1.6kb		+	+

Figure 3. Diagnostic key to hybridization patterns with *ILV3* probes against BglII-SalI restrictions of genomic DNA from *S. cerevisiae* and *S. carlsbergensis*. Probes 16-1 (i) and 12-1 (ii) are derived from two different alleles present in *S. carlsbergensis*.

2.5. Preparation of genomic DNA

Yeast genomic DNA was isolated based on the protocol of PEDERSEN (23). Stationary phase cells from 10 ml of YPD medium were suspended in 1 ml of 0.9 M-sorbitol, 0.1 M-EDTA pH 7.5, spun down at $15,000 \times g$ and resuspended in 0.4 ml of 0.9 M-sorbitol, 0.1 M-EDTA and 14 mM- β -mercaptoethanol. Zymolase-60000 (from *Arthrobacter luteus*, Seikagaku Kogyo Co. Ltd., Japan) was added (0.2 mg in 0.1 ml) and the mixture incubated at 37 °C for 20-30 minutes. After spheroplast formation was complete the cells were resuspended in 0.4 ml of TE and cell lysis induced by the addition of 90 μ l of 0.28 M-EDTA (pH 8.0), 0.4 M-Tris and 2.2% SDS. After 30 minutes at 65 °C, 80 μ l of 5 M-potassium acetate was added and the tube placed on ice for one hour. The tube was then spun for 15 minutes at $15,000 \times g$ and the nucleic acids in the supernatant precipitated with ethanol. The resulting precipitate was resuspended in 0.5 ml of TE and 25 μ l of 1 mg/ml RNase (Sigma R-5125) added. This was then incubated at 38 °C for 30 minutes followed by isopropanol precipitation of the DNA. The precipitate was then resuspended in 50 μ l of TE and 10-30 μ l of this was used per restriction enzyme analysis of genomic DNA.

2.6. *ILV3* and *CYC1* gene probes used in the molecular analysis of chromosome X

Two *ILV3* probes were used during the course of this study: (i) the 3.0 kb BamHI-EcoRI fragment from plasmid pGCT-16-1 and (ii) the 2.9 kb AvaI-SalI fragment from plasmid pGCT-12-1 (4). Both fragments contain functionally complete alleles of *ILV3* which were cloned from *S. carlsbergensis* 244 by CASEY (4). On the basis of restriction site mapping and Southern analysis the *ILV3* allele from pGCT-16-1 is known to be identical to the allele of *ILV3* found in *S. cerevisiae* S288C (4). The *ILV3* allele from pGCT-12-1, however, is unique to *S. carlsbergensis* and has an unrelated restriction site map. Each allele will only hybridize to the other under low stringency washing conditions (i.e. 60 °C, $3 \times \text{SSC}$). At 68 °C/ $0.1 \times \text{SSC}$ however, neither probe will hybridize to the other (4). In BglII-SalI digests of genomic DNA from *S. cerevisiae* S288C and recipient C85-0605 yeasts both probes hybridize to 6.8 and 3.9 kb fragments (pattern I) under low stringency conditions (4). In BglII-SalI digests of genomic DNA from parental and spore derived clones of *S. carlsbergensis* 244 yeast both probes hybridize to 6.8, 3.9, 3.9 and 1.6 kb fragments (pattern I and II) under low stringency conditions (4). At 68

$^{\circ}\text{C}/0.1\times\text{SSC}$ however the *ILV3* probe from pGCT-12-1 remains hybridized to the 3.9 and 1.6 kb fragments containing the allele unique to the lager yeast (pattern II) while only the *ILV3* probe from pGCT-16-1 remains hybridized to the 6.8 and 3.9 kb fragments containing the allele shared between *S. cerevisiae* and the lager yeast (4). Figure 3 depicts the hybridization patterns for these two probes.

The *CYC1* probe used in this study was a 430 base pair *XhoI* fragment from the promoter region of *CYC1* which had been cloned from *S. cerevisiae*. It was originally on the yeast-*E. coli* plasmid pRY65 (kindly provided by R. YOCUM, Biotechnica International, Cambridge, Mass.) and for the present study was sub-cloned before purification of the *XhoI* fragment for use as a probe. The fragment does not contain any *BglII*, *BamHI*, *EcoRI*, *SalI*, *XbaI*, or *HindIII* cleavage sites.

To isolate *ILV3* and *CYC1* probes, restriction fragments were first isolated from 0.7% (w/v) agarose gels by electroelution onto a dialysis membrane as outlined by YANG et al. (36). After ethanol precipitation the fragments were purified and concentrated using the Elutip-d system (Schleicher and Schuell Inc.) TAE buffer (40 mM-Tris, 20 mM-acetate, 2 mM- Na_2EDTA , and 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide), pH 8.5, was used as the electrophoresis buffer.

2.7. Chromosome separation electrophoresis

Electrophoresis apparatus and orthogonal-field-alternation gel electrophoresis conditions were set up according to CARLE and OLSON (3), while sample preparation and sample loading were based on the protocols of SCHWARTZ and CANTOR (29, 30) with the modifications described by PEDERSEN (22).

3. RESULTS

3.1. Transfer of chromosome X from spore derived clones of lager yeast *S. carlsbergensis* 244 to *S. cerevisiae* recipient strains

Brewers' yeasts, normally unable to mate and produce viable spores, are not suitable candi-

dates for chromosome transfer experiments (1, 9, 10). However, GJERMANSEN and SIGSGAARD (10) have sporulated the Carlsberg lager strain 244 and isolated viable spores exhibiting an α or α mating type (even though spore viability in general was extremely low). Three such meiotic segregants, C80-CG7, C80-CG65 and C80-CG110 were each crossed with the recipient *S. cerevisiae* C85-0605 strains of the opposite mating type listed in Table I. Mating mixtures were densely plated on minimal medium containing cycloheximide and adenine in order to select for C85-0605 recipient cells that had received at least *ARG3*, *MET3*, *ILV3*, and *HOM6* from the donor lager yeast (lysine was included in the selective medium for crosses involving *lys1* C85-0605 recipient strains). This medium does not permit the growth of either haploid parent or of true hybrids as the gene for cycloheximide resistance, *cyh2*, is recessive. Red colonies arose at a frequency of $1-2\times 10^{-6}$ per C85-0605 cell (white coloured colonies were discarded as they most probably represent normal diploids which by mitotic recombination in the *cyh2* locus result in homozygosity for *cyh2*).

Representative red colonies from each cross, which by virtue of their growth on this medium had received *ARG3*, *MET3*, *ILV3*, and *HOM6* from the meiotic segregant of the lager yeast (but not *ADE2* or *CYH2*), were then scored for the other two chromosome X markers of interest. Surprisingly, while all were found to be *CDC11*, clones derived from the same cross varied in the *RAD7* locus, scoring both as UV resistant and UV sensitive. This was true for crosses involving all three meiotic segregants of the lager yeast. Ten red colonies were then taken from each cross which retained the original mating type of the C85-0605 recipient strain and were not *LYS1* (to rule out co-transfer of chromosome IX). These were subsequently crossed with each of the tester *S. cerevisiae* C85-0605 strains of the opposite mating type listed in Table I and test dissections done on sporulated diploids in order to select for crosses yielding good spore viability. Four such crosses with high spore viability (65% to 82%) were selected for detailed analysis of chromosome X from the lager yeast.

Table III. Tetrad analysis of the cross between chromosome X transfer line C85-0605-A16 (*MAT α ARG3 MET3 ILV3 RAD7 CDC11 HOM6 ade2-40 cyh2 kar1-1*) and *S. cerevisiae* tester strain C85-0605-B2 (*MAT α arg3 met3 ilv3 cyc1-1 cdc11 hom6*)

	4:0	3:1	2:2	1:3	0:4
<i>ARG3/arg3</i>			35	4	
<i>MET3/met3</i>			39		
<i>ILV3/ilv3</i>			39		
<i>RAD7/cyc1-1</i>			39		
<i>CDC11/cdc11</i>		1 ^a	36	2 ^a	
<i>HOM6/hom6</i>			39		
	PD	NPD	TT	Distance (cM)*	
<i>arg3-met3</i>	22	2	11	32.8 cM	
<i>met3-ilv3</i>	39	0	0	--	
<i>ilv3-cyc1-1</i>	39	0	0	--	
<i>cyc1-1-cdc11</i>	37	0	0	--	
<i>cdc11-hom6</i>	37	0	0	--	

^a Interpreted to represent a meiotic gene conversion

* PERKINS formula used to calculate distances underestimates distances above 40 cM (24)

3.2. Tetrad analysis of recipient *S. cerevisiae* C85-0605 strains containing a chromosome X from *S. carlsbergensis*

The simultaneous transfer of *ARG3*, *MET3*, *ILV3*, *CDC11*, and *HOM6* suggested that most or all essential functions of chromosome X in *S.*

cerevisiae can be provided for by a single chromosome of *S. carlsbergensis*. As *RAD7* was not transferred in certain cases it suggested the presence of possibly two different chromosome X's in the lager yeast. To ascertain if this was the case, and to determine the degree of homology

Table IV. Tetrad analysis of the cross between chromosome X transfer line C85-0605-A29 (*MAT α ARG3 MET3 ILV3 RAD7 CDC11 HOM6 ade2-40 cyh2 kar1-1*) and *S. cerevisiae* tester strain C85-0605-A33 (*MAT α arg3 met3 ilv3 cyc1-1 cdc11 hom6*)

	4:0	3:1	2:2	1:3	0:4
<i>ARG3/arg3</i>			25	1 ^a	
<i>MET3/met3</i>			26		
<i>ILV3/ilv3</i>			26		
<i>RAD7/cyc1-1</i>			26		
<i>CDC11/cdc11</i>			26		
<i>HOM6/hom6</i>			26		
	PD	NPD	TT	Distance (cM)*	
<i>arg3-met3</i>	12	0	13	26.0 cM	
<i>met3-ilv3</i>	26	0	0	--	
<i>ilv3-cyc1-1</i>	26	0	0	--	
<i>cyc1-1-cdc11</i>	26	0	0	--	
<i>cdc11-hom6</i>	26	0	0	--	

^a Interpreted to represent a meiotic gene conversion

* PERKINS formula used to calculate distances underestimates distances above 40 cM (24)

Table V. Tetrad analysis of the cross between chromosome X transfer line C85-0605-C2 (*MAT α ARG3 MET3 ILV3 RAD7 CDC11 HOM6 ade2-40 cyh2 kar1-1*) and *S. cerevisiae* tester strain C85-060585-B10 (*MAT α arg3 met3 ilv3 cyc1-1 cdc11 hom6*)

	4:0	3:1	2:2	1:3	0:4
<i>ARG3/arg3</i>			47	3 ^a	1 ^b
<i>MET3/met3</i>		1 ^a	47		
<i>ILV3/ilv3</i>		1 ^a	47		
<i>RAD7/cyc1-1</i>		1 ^a	47		
<i>CDC11/cdc11</i>		2 ^a	46		
<i>HOM6/hom6</i>		1 ^a	47		
	PD	NPD	TT	Distance (cM)*	
<i>arg3-met3</i>	13	1	29	40.7 cM	
<i>met3-ilv3</i>	47	0	0	--	
<i>ilv3-cyc1-1</i>	47	0	0	--	
<i>cyc1-1-cdc11</i>	46	0	0	--	
<i>cdc11-hom6</i>	46	0	0	--	

^a Interpreted to represent a meiotic gene conversion^b Interpreted to represent a mitotic gene conversion

* PERKINS formula used to calculate distances underestimates distances above 40 cM (24)

between chromosome X in *S. cerevisiae* and *S. carlsbergensis* lager yeast, recombination studies were carried out on crosses between tester *S. cerevisiae* C85-0605 strains and four chromosome X transfer lines of C85-0605 (three of which were *RAD7* and one which was UV sensitive).

Tables III, IV, and V contain tetrad analysis data from crosses involving chromosome X transfer lines which received all chromosome X markers from the lager yeast (namely C85-0605-A16, C85-0605-A29, and C85-0605-C2). In all three crosses a 2:2 segregation was seen for the six chromosome X markers. From the linkage data (shown in the lower part of the tables) it can be seen that *ARG3*, *MET3*, *ILV3*, *RAD7*, and *HOM6* are linked in the lager yeast chromosome involved in these crosses, confirming it to contain all of the essential functions of the markers tested in chromosome X from *S. cerevisiae*. The structure of these chromosomes is quite different however as recombination between the chromosome X in the tester C85-0605 strains and the C85-0605 chromosome transfer lines was only observed between the *arg3* and *met3* loci. All of the markers on the right arm segregated as if centromere linked and as recombination was

only seen in the left arm for all three crosses it suggests that the chromosome transfer lines C85-0605-A16, C85-0605-A29, and C85-0605-C2 contain the same chromosome X from the lager yeast. Distances between the *arg3-met3* loci ranged from 26.0 cM (Table IV) to 40.7 cM (Table V), considerably shorter than the 60.6 cM value reported in *S. cerevisiae* (18).

Tables VI and VII contain tetrad analysis data from crosses involving chromosome X transfer line C85-0605-M31 which received all chromosome X markers from lager yeast C80-CG65 except for *RAD7*. Examining Table VI first, it can be seen that the markers predominantly segregated 2:2 and that all of the chromosome X markers tested were linked. Once again recombination between the chromosomes X of the tester and transfer strains was limited (suggesting different chromosome structure) but in this case recombination was only detected in the right arm of chromosome X (in the region spanning *ilv3-hom6*). Therefore, in terms of the loci contained, not only is the chromosome X from the lager yeast in C85-0605-M31 different from the chromosome X in the other three chromosome transfer strains, but the recombination data reveal it to be different also in terms

Table VI. Tetrad analysis of the cross between the UV sensitive chromosome X transfer line C85-0605-M31 (*MAT α ARG3 MET3 ILV3 CDC11 HOM6 ade2-40 cyh2 kar1-1*) and *S. cerevisiae* tester strain C85-0605-B19 (*MAT α arg3 met3 ilv3 cycl-1 cdc11 hom6*) from asci with four viable spores

	4:0	3:1	2:2	1:3	0:4
<i>ARG3/arg3</i>			29		
<i>MET3/met3</i>			28	1 ^a	
<i>ILV3/ilv3</i>			28	1 ^a	
<i>CDC11/cdc11</i>			26	3 ^a	
<i>HOM6/hom6</i>			24	5	
	PD	NPD	TT	Distance (cM)*	
<i>arg3-met3</i>	28	0	0	--	
<i>met3-ilv3</i>	28	0	0	--	
<i>ilv3-cdc11</i>	24	1	0	4 cM	
<i>cdc11-hom6</i>	17	0	5	11.4 cM	

^a Interpreted to represent a meiotic gene conversion

* PERKINS formula used to calculate distances underestimates distances above 40 cM (24)

Table VII. Tetrad analysis of the cross between the UV sensitive chromosome X transfer line C85-0605-M31 (*MAT α ARG3 MET3 ILV3 CDC11 HOM6 ade2-40 cyh2 kar1-1*) and *S. cerevisiae* tester strain C85-0605-B19 (*MAT α arg3 met3 ilv3 cycl-1 cdc11 hom6*) from asci with three viable spores*

	PD	NPD	TT	Distance (cM)*
<i>arg3-met3</i>	48	0	0	--
<i>met3-ilv3</i>	48	0	0	--
<i>ilv3-cdc11</i>	29	1	18	25.0 cM
<i>cdc11-hom6</i>	31	2	13	27.2 cM

* A 2:2 segregation was assumed

of the capacity for crossing over.

The high frequency of 1:3 segregation for *HOM6:hom6* (17%) and *ARG3:arg3* (11%) in the crosses involving C85-0605-M31 and C85-0605-A16 respectively, indicate that these strains are chromosome addition lines. This notion was subsequently confirmed for both of these strains by electrophoretic chromosome separation (cf. Figure 9).

A surprising observation during tetrad analysis of the C85-0605-M31 cross was that spores from asci in which only three of the spores were viable exhibited more extensive recombination in the *ilv3-cdc11* and *cdc11-hom6* regions (Table VII) than in asci with four viable spores. It therefore would appear that recombination in this segment between the tester chromosome X and the chromosome X of the lager yeast result-

Figure 4. Southern hybridization analysis of BglII-SalI cleaved genomic DNA under low (60 °C, 3 $\times$ SSC) and high (68 °C, 0.1 $\times$ SSC) stringency conditions when probed with the *ILV3* alleles of (a) pGCT-12-1 and (b) pGCT-16-1. The same filter was used for both probes. Lane A, chromosome X substitution line C85-0605-C2; lane B, chromosome X addition line C85-0605-A16; lane C, chromosome X substitution line C85-0605-A29; lane D, chromosome X addition line C85-0605-M31; lane E, *S. carlsbergensis* 244; lane F, *S. cerevisiae* C85-0605-B40; lane G, lambda-DNA cleaved with HindIII; lane H, lambda-DNA cleaved with EcoRI.

a) (i) Low Stringency (60°C, 3XSSC) (ii) High Stringency (68°C, 0.1XSSC)

b) (i) Low Stringency (60°C, 3XSSC) (ii) High Stringency (68°C, 0.1XSSC)

a) Low Stringency (60°C, 3XSSC)

A B C D E F G H I J K L M N O P Q R

b) High Stringency (68°C, 0.1XSSC)

A B C D E F G H I J K L M N O P Q R

Figure 5. Southern hybridization analysis of BglII-Sall cleaved genomic DNA from recipient *S. cerevisiae* C85-0605-A5 clones containing a *S. carlsbergensis* chromosome X from C80-CG7 which can complement *arg3 met3 ilv3 rad7 cdc11* and *hom6*. The *ILV3* allele of pGCT-12-1 was used as a probe under (a) low (60 °C, 3×SSC) and (b) high (68 °C, 0.1×SSC) stringency conditions. Lanes A-L, C85-0605-A1, 2, 6, 7, 11, 19, 24, 27, 28, 33, 38, and 39, respectively; lane M, C85-0605-A5; lane N, *S. carlsbergensis* 244; lane O, C80-CG65; lane P, C80-CG110; lane Q, HindIII cleaved lambda-DNA; lane R, EcoRI cleaved lambda-DNA.

ed in a chromatid making one spore being inviable. Further experiments are needed to clarify the genetic basis for this observation.

3.3. Southern analysis of chromosome X transfer lines C85-0605-A16, C85-0605-A29, C85-0605-C2, and C85-0605-M31 with *ILV3* gene probes cloned from the Carlsberg lager strain 244

The genetic data in Tables III-VII indicates that there are at least two chromosomes X in *S. carlsbergensis* which not only differ considerably from chromosome X in *S. cerevisiae*, but from each other as well. To obtain information on the degree of sequence homology between these chromosomes as regards the *ILV3* locus, as well as to determine if the genetically analyzed clones are substitution or addition lines, the four strains were probed with two *ILV3* gene probes from the parental lager strain (described in detail in the Materials and Methods, 2.5).

Figure 4 depicts Southern hybridization patterns of BglII-Sall digestions of genomic DNA from the four chromosome X transfer lines (lanes A-D), the Carlsberg lager yeast strain (lane E) and a reference recipient *S. cerevisiae* strains (C85-0605-B40, lane F). The C85-0605-A29 and C85-0605-C2 strains exhibited pattern II hybridization (Figure 3) with the *ILV3* probes indicating that they are chromosome X substitution lines (the recipient C85-0605 strain exhibits pattern I with these probes). The C85-0605-A16 strain, however, is an addition line for chromosome X as it exhibits both pattern I and II hybridization. Only strain C85-0605-M31 had a chromosome X which exhibited pattern I hybridization.

The results in Figure 4 indicate that when all chromosome X markers are transferred (i.e.

C85-0605-A16, C85-0605-A29, C85-0605-C2) the chromosome involved contains the allele of *ILV3* unique to the lager yeast (4). In the case of C85-0605-M31, however, where the UV sensitive phenotype of *cyc1-1* was not complemented, the chromosome involved contained the allele of *ILV3* common to both *S. cerevisiae* S288C and the lager yeast. To determine if this observation remained valid under wider testing, genomic DNA preparations from a large number of chromosome X transfer lines, for which no tetrad analysis data was available, were probed with the two *ILV3* gene probes. Figure 5 examines twelve clones from cross C85-0605-A5 × C80-CG7 (in which all chromosome X markers were transferred) probed with the

Figure 6. Southern hybridization analysis of genomic DNA from *S. cerevisiae* recipient strains containing a chromosome X from *S. carlsbergensis* which could complement all chromosome X markers except for *rad7*. The probe was the *ILV3* allele found in pGCT-12-1 and low stringency conditions were used (60 °C, 3×SSC). Lanes A-O are chromosome X transfer lines C85-0605-A78, -C48, -L100, -M44, -N14, -M13, -B6, -C63, -L28, -M27, -N20, -C87, -K12, -L98, and -M36, respectively. Lane P, *S. carlsbergensis* 244. Lane Q, *S. cerevisiae* C85-0605-B40. Lane R, HindIII cleaved DNA.

a) Low Stringency (60°C, 3XSSC)

b) High Stringency (68°C, 0.1XSSC)

Figure 7. Southern hybridization analysis of *S. cerevisiae* S288C, *S. carlsbergensis* C80-CG7 and CG65 and *S. cerevisiae* C85-0605 lines with an acquired carlsbergensis chromosome X using a *CYC1* gene probe from *S. cerevisiae*. Hybridization was carried out under (a) low stringency (60 °C, 3×SSC) and (b) high stringency (68 °C, 0.1×SSC) conditions. Lanes A-H are BglII digests of genomic DNA from *S. cerevisiae* S288C, *S. carlsbergensis* C80-CG7, *S. carlsbergensis* C80-CG65 and chromosome X transfer lines C85-0605-C48, -C87, -M31 (chromosome X addition line), -M44, and -M13, respectively. Lanes I, J, and K; L, M, and N; O, P, and Q are EcoRI, SalI and XbaI digests of genomic DNA from *S. cerevisiae* S288C, *S. carlsbergensis* C80-CG7 and *S. carlsbergensis* C80-CG65, respectively. Lane R, HindIII cleavage of lambda-DNA, Lane S, EcoRI and HindIII cleavage of lambda-DNA.

pGCT-12-1 allele of *ILV3* which is unique to *S. carlsbergensis*. Once again only pattern II hybridization was found under high stringency washing conditions (b) indicating that they all contain the chromosome X from the lager yeast containing the unique *ILV3* allele. Chromosome X substitution occurred at a high frequency; eight of the twelve clones were substitution lines as they did not display pattern I + II hybridization under low stringency conditions (a). In Figure 6, 15 clones from crosses involving all three lager yeast meiotic segregants, but in which the *RAD7* marker was not transferred, were probed with *ILV3* from pGCT-12-1. As was seen in the case of C85-0605-M31, all fifteen exhibited pattern I hybridization under low stringency conditions, indicating that all had a chromosome X from the lager yeast containing the allele of *ILV3* common to both *S. cerevisiae* S288C and *S. carlsbergensis*. As the hybridization pattern of substitution and addition lines involving this type of chromosome X would be identical, it is not possible to reach any conclusions regarding the frequency of chromosome substitution.

3.4. Southern analysis of the *CYC1* locus on chromosome X

As the ability to complement the UV sensitive phenotype of the *cyc1-1* mutation was not transferred in C85-0605-M31 and in the clones depicted in Figure 6, the *CYC1* locus in several of these lines was compared with that in the lager yeast using a *CYC1* probe from *S. cerevisiae* (Figure 7). Once again the lager yeasts were found to contain two alleles of a gene in chromosome X – one which is located on the same hybridizing fragments in BglII (lanes A-H),

EcoRI (lanes I-K), SalI (lanes L-N), and XbaI (lanes O-Q) digests of *S. cerevisiae* S288C DNA, and one which is unique to the lager yeasts. BglII digests of genomic DNA in Figure 7a reveals a hybridizing fragment of 4.5 kb that is common to *S. cerevisiae* S288C (lane A), C80-CG7 (lane B), C80-CG65 (lane C) and chromosome transfer lines C85-0605-C48, -C87, -M31, -M44, and -M13 (lanes D-H). The chromosome transfer lines lack the 4.8 kb fragment that is unique to the lager yeast i.e. only the chromosome X containing the *CYC1* allele in common with that found in *S. cerevisiae* was transferred into the clones which remained UV sensitive. This is consistent with earlier observations with *ILV3* where only the chromosome containing the allele of *ILV3* in common with *S. cerevisiae* lacked *rad7* complementing ability.

It is interesting to note that an XbaI site exists in the allele of *CYC1* that is unique to C80-CG7 and -CG65 (lanes P and Q, respectively). This shows differences in the nucleotide sequences of these two alleles. However, in general the degree of sequence homology is considerably higher in the *CYC1* alleles than in the *ILV3* alleles as increasing the stringency of the washing conditions (Figure 7b) resulted in a much less noticeable decline in the hybridization intensity of the fragment unique to *S. carlsbergensis* than is seen with the *ILV3* alleles. The lack of *RAD7* in the *CYC1* region of *S. carlsbergensis* is accordingly not due to a deletion comparable with that of *cyc1-1*.

3.5. Electrophoretic chromosome separation with chromosome X transfer lines

Based on tetrad analysis and Southern hybridization data in sections 3.2 and 3.3 it is not

Figure 8. Photograph of a gel in which the chromosome sized DNA molecules of chromosome X addition and substitution lines were separated by field alternation electrophoresis. The autoradiographs of the gel probed with the two *ILV3* alleles of *S. carlsbergensis* 244 are given in Figure 9. Lanes A and C are *S. carlsbergensis* C80-CG7 and *S. cerevisiae* C85-0605-B40, the donor and recipient strains respectively for the chromosome X substitution line *S. cerevisiae* C85-0605-C2 (lane B). Lanes D and I are chromosome X substitution and addition lines *S. cerevisiae* C85-0605-A29 and -A16, respectively arising from the cross between *S. carlsbergensis* C80-CG7 (lane A) and *S. cerevisiae* C85-0605-A5 (lane H). Lanes E and G are chromosome X addition lines *S. cerevisiae* C85-0605-M31 (the strain in lane G has a deeper red colony colour than the original isolate) arising from the cross between *S. carlsbergensis* C80-CG65 (lane K) and *S. cerevisiae* C85-0605-A10 (not shown). Lane F is *S. cerevisiae* K5-5A, used as a reference strain to locate chromosome X. Lane J is addition line C85-0605-A16 (also shown in lane I). Electrophoresis was carried out for 18 hours at 200 mA, 300V, 5-8 °C with a switching time of 50 seconds.

possible to conclude whether a clone is an addition or substitution line if it contains the chromosome X from *S. carlsbergensis* lacking *RAD7*. Southern analysis of blotted chromosome separations however does make it possible to reach such conclusions as depicted in Figures 8 and 9. Figure 8 is a photograph of the chromosome separation gel that was probed in Figure 9 with the two *ILV3* alleles of Carlsberg lager strain 244. In it, substitution clones C85-0605-C2

(lane B) and C85-0605-A29 (lane D) can clearly be seen to have substituted chromosome X from the recipient C85-0605 strains (lanes C and H, respectively) with a more slowly migrating chromosome X from C80-CG7 (lane A). The addition line C85-0605-A16, however, (lane I) has two chromosomes which hybridize to the *ILV3* probes under low stringency conditions – the original chromosome X from the recipient (lane H) and the more slowly migrating chromosome X of C80-CG7. These results confirm the earlier speculation that these three chromosome transfer lines contain the same chromosome X from *S. carlsbergensis* C80-CG7. The strong degree of hybridization of the *ILV3* probe from pGCT-12-1 to these lines under high stringency conditions (Figure 4) also confirms that this chromosome contains the allele of *ILV3* unique to *S. carlsbergensis*. It is worthwhile to note that chromosome X was the only chromosome transferred from the lager yeasts in these crosses. The chromosome profile of the addition and substitution lines only differs at the chromosome X location, with no new bands or doublets being observed (relative to the original recipient *S. cerevisiae* C85-0605 strains).

In the case of the C85-0605-M31 strain, however, a different chromosome X was transferred from the lager yeast (as predicted from the genetic and Southern data). Lanes E and G depict C85-0605-M31 (the isolate in lane G had a deeper red colony colour than that in lane E) and in this case a more rapidly migrating chromosome X (donated from C80-CG65, lane K) had been transferred to the recipient C85-0605-A10 strain (not shown). As C85-0605-M31 contains two hybridizing chromosomes at low stringency conditions, it can now be concluded to be an addition line for chromosome X. This chromosome, lacking *RAD7*, contains the allele of *ILV3* that is found in *S. cerevisiae* yeast as only the *ILV3* probe from pGCT-16-1 remains bound to this chromosome under high stringency washing conditions (Figure 9b).

A surprising observation in Figure 9 is that lager yeast C80-CG65 has three chromosomes which hybridize to the *ILV3* probes under low stringency conditions. C80-CG7, however, only has two, suggesting that these meiotic segregants differ in their genetic composition. It also indi-

Figure 9. Southern hybridization analysis of the gel with the electrophoretically separated chromosomes shown in Figure 8. The probes used were the *ILV3* allele of pGCT-12-1 (a) and pGCT-16-1 (b) under low (60 °C, 3×SSC) and high (68 °C, 0.1×SSC) stringency conditions. Lane designations are the same as those used in Figure 8. The same filter was used for both *ILV3* probes.

cates that the parental *S. carlsbergensis* 244 lager strain is trisomic for chromosome X. This idea was confirmed in a later chromosome separation experiment (Figure 10) where a Southern analysis of the *S. carlsbergensis* 244 parent strain (lane D) and C80-CG110 and C80-CG65 meiotic segregants (lanes A and B) clearly reveals the presence of three hybridizing chromosomes X. It is therefore apparent that the middle migrating chromosome X has yet to be isolated and analyzed in the background of a *S. cerevisiae* recipient strain.

4. DISCUSSION

The relatively new technique of chromosome separation electrophoresis is a powerful tool for industrial mycologists as it is now possible to visualize yeast chromosomes in a gel and, by

utilizing gene probes for specific chromosomes, determine chromosome ploidy. This report presents direct evidence that lager brewing yeasts are heteroploid. While brewing yeasts were widely believed to be polyploid (7, 16, 28, 32), the evidence used to support these claims has largely been indirect or based on techniques known to be difficult to interpret, e.g. on the basis of segregation of mating and sporulation phenotype (7) or measurement of DNA content per cell (16, 28, 32).

Using the *ILV3* alleles of *S. carlsbergensis* (Carlsberg lager strain 244) as chromosome probes, this lager strain was shown to be trisomic for chromosome X (assuming no co-migration of an additional chromosome X). The three chromosomes migrate independently of each other during chromosome separation electrophoresis and two of these have been isolated

Figure 10. Southern hybridization analysis of chromosome blots probed with the *ILV3* allele of pGCT-12-1 under low stringency conditions. Lane A, *S. carlsbergensis* C80-CG110; lane B, *S. carlsbergensis* C80-CG65; lane C, *S. cerevisiae* C85-0605-M31; lane D, *S. carlsbergensis* 244; lane E, *S. cerevisiae* K5-5A.

and analyzed in the genetic background of *S. cerevisiae*. Type I chromosome X migrates more slowly than the reference chromosome X of *S. cerevisiae* K5-5A and contains the allele of *ILV3* unique to the lager yeast. It can recombine with chromosome X from *S. cerevisiae*, but only in the left arm. Type II chromosome X migrates faster than the chromosome X of *S. cerevisiae* K5-5A and contains the allele of *ILV3* that is shared between the lager yeast and *S. cerevisiae* S288C. It also can recombine with chromosome X from *S. cerevisiae*, but in this case only in the right arm of chromosome X. Both type I and type II chromosomes X are different homeologues of chromosome X when compared with that of *S. cerevisiae*. A similar situation has been found with chromosomes III and V from this yeast (19, 21, 34), but with chromosomes XII

and XIII both homologues and homeologues to the corresponding chromosomes in *S. cerevisiae* have been reported (34). It is interesting to note however that type III chromosome X in the lager yeast co-migrates with chromosome X from the reference *S. cerevisiae* K5-5A yeast. Efforts are currently under way to isolate and analyze this chromosome in order to determine if it is homologous to the chromosome X of *S. cerevisiae* (as *S. carlsbergensis* 244 seems to contain only two alleles of *ILV3* (4) this chromosome is not expected to contain a novel allele of *ILV3*). It will also be interesting to conduct electrophoretic chromosome separation and molecular hybridization experiments involving chromosomes III, V, XII, and XIII in order to determine if a third type exists for these chromosomes as well.

Carlsberg lager strain 244 exhibits the normal properties of lager brewing yeasts which makes their genetic manipulation difficult i.e. poor mating ability, poor sporulation and poor spore viability (10). The production of aneuploid spores, which would result in poor spore viability (7), can now be concluded to contribute to these properties as spore derived clones from this yeast were found to be either disomic (as in C80-CG7) or trisomic (as in C80-CG65 and -CG110) for chromosome X. The demonstrated nucleotide sequence inhomology between the different types of chromosome X will also contribute to these properties as recombination is a prerequisite for proper disjunction of chromosomes in meiosis. Ultimately, it is hoped that by increasing our understanding of the genetic composition of lager yeast sophisticated breeding programs can be facilitated.

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