CLONING AND ANALYSIS OF TWO ALLELES OF THE *ILV3* GENE FROM *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

---

Keywords: Lager yeast, dihydroxyacid dehydrase, isoleucine, valine, genomic library, cloning, diacetyl, chromosome X

A genomic library of a *Saccharomyces carlsbergensis* lager's yeast DNA was constructed in the yeast-*E. coli* shuttle vector YRp17. Two alleles of the *ILV3* gene were cloned from the library by complementation of the *ilv3-12* mutation in strains of *Saccharomyces cerevisiae* yeast. Restriction site mapping and Southern hybridisation using an *ILV3* probe from *Saccharomyces cerevisiae* S288C revealed one allele from the lager yeast to be closely related, or identical, to the *ILV3* gene in *S. cerevisiae* S288C. The second allele has a different restriction site map and limited sequence homology with the *ILV3* gene of *S. cerevisiae* S288C. The implications of these results in determining the genetic constitution of lager yeast and on research programs designed to genetically engineer lager yeast are discussed.

1. INTRODUCTION

The biosynthetic pathways for the branched-chain amino acids isoleucine and valine in *Saccharomyces cerevisiae* are catalyzed by five enzymes (19, 23), as shown in Figure 1. Threonine deaminase, the first enzyme in the pathway leading to isoleucine biosynthesis, is an allosteric enzyme which converts threonine to α -ketobutyrate. The remaining four steps, which convert pyruvate to isoleucine and valine, share the same enzymes – an unusual occurrence in biosynthetic pathways. In addition, the third branched-chain amino acid, leucine, is synthesized in a series of four reactions with α -ketoisovalerate (the last intermediate in the synthesis of

valine) as the starting point (36). With the exception of the transaminase, all enzymes of the pathways are believed to be located in the mitochondria (35).

The synthesis of the enzymes in these pathways is regulated by two control mechanisms. The first, a pathway-specific control mechanism, is analogous to multivalent repression in bacteria (14) where synthesis of the enzymes is decreased in the presence of isoleucine, valine, and leucine (5, 22). Under the second mechanism, general amino acid control, the synthesis of threonine deaminase (16) and transaminase (9) enzymes is derepressed in response to amino acid starvation.

Abbreviations: EDTA = ethylene diamine tetraacetic acid, sodium salt; kb = kilo base; SC = synthetic complete medium; SDS = sodium dodecyl sulphate; SSC = 0.15 M-NaCl, 15 mM-Na citrate, pH 7.0; Tris = tris(hydroxymethyl)-amino methane

Figure 1. The biosynthetic pathway of isoleucine and valine in *Saccharomyces cerevisiae*.

In *Saccharomyces cerevisiae* the structural genes coding for the first four steps of the pathways are known (19, 23). However, no gene has been identified for the transaminase enzyme, either because mutations in this gene may be lethal or because transaminases encoded by more than one gene can catalyze the reactions. The four genes have been mapped in *Saccharomyces cerevisiae* with *ILV1* (coding for threonine deaminase), *ILV2* (coding for acetohydroxyacid synthetase), *ILV5* (coding for acetohydroxyacid reductoisomerase), and *ILV3* (coding for dihydroxyacid dehydrase) being located on chromosomes V, XIII, XII, and X, respectively (26, 32). All four genes from *S. cerevisiae* (12, 31, 33) have been cloned, and the nucleotide sequences of *ILV1* (20) and *ILV2* (11) determined.

The molecular genetics of the isoleucine-valine pathways in *Saccharomyces* is of considerable interest to the brewing industry (39). A desirable improvement in brewers' yeast is the construction of strains which produce little or no off-flavour compounds – several of which, espe-

cially diacetyl, are formed from intermediates of these pathways (6). Until recently, progress in this area was minimal due to the lack of knowledge of the genetic constitution of brewers' yeast which, unlike genetic standard strains of *Saccharomyces cerevisiae*, exhibit no or poor sporulation ability, low spore viability, and rare expression of mating type (1, 13, 18, 38, 40). This has made genetic analysis and directed improvement of these yeasts by traditional methods virtually impossible.

Recently, the isolation of spore clones expressing mating type in combination with the technique of single chromosome transfer has permitted studies of genes on chromosomes III, V, XII, and XIII of *Saccharomyces carlsbergensis* (15, 27, 39) by complementation, recombination, and molecular analysis. The brewer's yeast was found to be at least disomic for these chromosomes and to contain two versions of them. From a functional viewpoint the two chromosomes can complement *S. cerevisiae* chromosomes with several biochemical mutations and thus must carry the same genes, but

Table I. *Saccharomyces cerevisiae* *ilv3* mutants used in complementation experiments to clone the *ILV3* alleles of *Saccharomyces carlsbergensis* strain 244.

Strain	Genotype	Source
C80-1133	<i>MATα trp1 his4 arg4 lys1 ura4 ilv3-33</i>	J.G.L. PETERSEN
C80-1153	<i>MATα trp1 his4 arg4 lys1 ura4 ilv3-53</i>	J.G.L. PETERSEN
IVPX5-2B	<i>MATα his leu2 ura3 ilv3-12</i>	J. POLAINA (33)

they differ in the nucleotide sequence to the extent that recombination is drastically altered (15, 27, 39). Accordingly, the *Saccharomyces carlsbergensis* alleles display restriction endonuclease site polymorphisms in molecular hybridization studies using radioactively labelled *Saccharomyces cerevisiae* gene probes. Examples include *HIS4* (15, 27, 29), *LEU2* (15, 30), and regions adjacent to *MAT* (15) on chromosome III and *ILV1*, *URA3*, and *CYC7* on chromosome V (28). In the case of the *RDN1* gene on chromosome XII, no gene polymorphism has yet been uncovered in *Saccharomyces carlsbergensis*, which suggests that chromosomal organization and nucleotide sequences of this gene is very similar to that in *Saccharomyces cerevisiae*.

This paper examines gene polymorphisms in the *ILV3* locus of *Saccharomyces carlsbergensis*.

A genomic library of a *S. carlsbergensis* lager brewing strain has been constructed and used to clone genes involved in the isoleucine-valine pathways. As will be demonstrated, *Saccharomyces carlsbergensis* brewer's yeast contains two alleles of the *ILV3* gene in its genome. From Southern analysis and restriction site mapping one is shown to have a high degree of sequence homology to the *ILV3* allele found in *Saccharomyces cerevisiae*, while the second has low sequence homology to the *S. cerevisiae* *ILV3* allele of genetic standard strains. Eventually, such discoveries will assist in the development of research programs to genetically engineer and improve the industrial properties of *Saccharomyces* brewers' yeasts.

2. MATERIALS AND METHODS

2.1. Strains

A production lager strain of *Saccharomyces carlsbergensis* 244 from the Carlsberg Breweries was used as the source of donor DNA in the construction of the genomic library. Strains of *Saccharomyces cerevisiae* used to clone alleles of *ILV3* by complementation are listed in Table I.

Escherichia coli HB101 (4) was used as the recipient strain in bacterial transformation experiments and for the isolation and amplification of plasmid DNA.

2.2. Media and growth conditions

Escherichia coli HB101 was grown at 37 °C in LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, and 0.1% (w/v) glucose) containing either ampicillin (50 µg/ml) or tetracycline (15 µg/ml) as required.

Yeast strains were grown at 30 °C on synthetic complete medium, SC, as described by PETERSEN et al. (41). Selective media involved the

Figure 2. Restriction site map of the yeast - *Escherichia coli* shuttle vector YRp17. The location of the cleavage sites for *Ava*I, *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Pst*I, *Pvu*I, *Pvu*II, *Sal*I, and *Xba*I are shown. The black line corresponds to *TRP1*, *URA3*, and *ARS* (origin of replication) genes from *Saccharomyces cerevisiae*. The stipled line is pBR322 DNA.

omittance of specific nutrients from SC medium (i.e., SC-ura-his-leu-ile-val is SC medium without uracil, histidine, leucine, isoleucine, and valine).

as well as genes conferring resistance to ampicillin and tetracycline in *Escherichia coli*. YRp17 was also used for subcloning experiments.

2.3. Cloning vector

The cloning vector, shown in Figure 2, was the yeast - *Escherichia coli* shuttle vector YRp17 (3) kindly supplied by M McDONELL. It contains the *TRP1* and *URA3* genes for selection in yeast

2.4. Construction of a genomic library of *Saccharomyces carlsbergensis* 244

Total yeast DNA was purified by the guanidinium chloride method (21) from strain 244 of *Saccharomyces carlsbergensis*. The DNA was

Figure 3. Agarose gel electrophoresis of DNA from four different plasmid pools. Lanes A, B, C, and D are undigested DNA from pools Nos. 1, 2, 3, and 4, respectively. Lanes E, F, G, and H are BamHI digestions of DNA from pools Nos. 1, 2, 3, and 4, respectively. Lane I is undigested YRp17 DNA. Lane J is HindIII digested λ -DNA.

partially digested with the restriction endonuclease *Sau* 3AI (New England Biolabs, Inc.) and 3-10 kb fragments were isolated by sucrose density gradient centrifugation as outlined by MANIATIS et al. (25). YRp17 was linearized with *Bam*HI, treated with calf alkaline phosphatase (Boehringer-Mannheim) to prevent recircularization and then mixed with an equal amount of the 3-10 kb *Saccharomyces carlsbergensis* 244 DNA fragments. After treatment with T4 DNA ligase (Boehringer-Mannheim) for 18 hours at 12 °C, the resulting ligation mixture was used to transform *Escherichia coli* HB101 cells made competent by the CaCl_2 procedure (24). At least 95% of the resulting transformants were ampicillin resistant/tetracycline sensitive indicating that their plasmids contained *Saccharomyces carlsbergensis* inserts. Ten randomly picked ampicillin resistant, tetracycline sensitive clones were screened for their plasmid content and found to contain recombinant plasmids with inserts ranging from 3 to 15 kb. A total of 18,000 independent transformants were generated and pools of approximately 2,000 colonies each were prepared by washing colonies off LB + ampicillin plates for storage at -80 °C in 60% (v/v) LB + 40% (v/v) glycerol. Assuming that *Saccharomyces carlsbergensis* strain 244 is a diploid (with a genome size of 2.8×10^4 kb), and that the average size of the inserts is 5.0 kb there is a 95% probability that this genomic library will contain any genomic DNA sequence (25). Large amounts of recombinant plasmid DNA were prepared by a scaled up version of the alkaline extraction procedure of BIRNBOIM and DOLY (2) followed by cesium chloride equilibrium centrifugation. Plasmid samples from pools were digested with *Bam*HI and electrophoresed in agarose gels (0.7% (w/v)) in order to estimate the size of the inserts (Figure 3). TAE buffer (40 mM-Tris base, 20 mM-acetic acid, 2 mM- Na_2EDTA , and 0.5 $\mu\text{g/ml}$ ethidium bromide), pH 8.5, was used as the electrophoresis buffer. These were found to contain a spectrum of *Saccharomyces carlsbergensis* inserts primarily corresponding to the 3-10 kb range expected.

2.5. Cloning and subcloning of *ILV3*

Recombinant plasmid DNA preparations were used to transform LiNO_3 treated *Saccha-*

romyces cerevisiae IVPX5-2B cells as described by ITO et al. (17). Clones which had taken up recombinant plasmids were first isolated on plates of SC-ura. Upon replica plating to SC-ura-ile-val, clones with plasmids containing an *ILV3* gene were identified by complementation of the *ilv3-12* mutation. Plasmids from such clones were isolated as described by DAVIS et al. (8), amplified in *Escherichia coli* HB101 and cesium chloride plasmid preparations produced for subsequent characterization.

In subcloning experiments DNA restriction fragments were isolated from 0.7% (w/v) agarose gels by electroelution onto a dialysis membrane as outlined by YANG et al. (41). After ethanol precipitation the fragments were purified and concentrated using the ELUTIP-d system (Schleicher and Schuell Inc.).

2.6. Restriction site mapping

Restriction endonucleases were obtained from Boehringer Mannheim and used according to the protocol of MANIATIS et al. (25). The only exception was *Kpn*I which was found to digest better under the conditions recommended by the manufacturer. In Figures 4 and 5, only those vector cleavage sites are depicted which were actually tested for in the recombinant plasmid.

2.7. Southern hybridization

After restriction endonuclease treatment, fragments of total yeast DNA or recombinant plasmid DNA were electrophoretically separated on 0.7% (w/v) agarose gels and then transferred to nitrocellulose filters (Millipore HAWP 000 10) by a modification of the method of SOUTHERN (37) as described by MANIATIS et al. (25). Probes consisted of linear restriction fragments (purified in the same manner as described for subcloning in section 2.5) containing all or part of an *ILV3* allele. They were labelled in vitro with [α - ^{32}P]dATP (New England Nuclear) by nick translation according to RIGBY et al. (34) using DNA polymerase I (Boehringer Mannheim) at 14 °C for 90 minutes. Unincorporated nucleotides were separated from the probe by centrifugation on a Sephadex G-50 (Pharmacia) column.

Figure 4. Restriction site maps of the *ilv3* complementing plasmids pGCT-16-1, pGCT-22-1, and pGCT-6/10. Plasmid pGCT-6/10 is a BamHI-EcoRI subclone of pGCT-16-1. The thick line is vector DNA and the thin line is *Saccharomyces carlsbergensis* 244 DNA. The dotted lines represent regions of the inserts with homology to the pE30/5-6-3 *ILV3* probe and the dashed lines represent regions which by Southern analysis (section 3.7) are deduced to be contiguous genomic DNA.

Molecular hybridization of labelled probes to filter bound DNA was done in plastic trays containing 100 ml of hybridization fluid (3×SSC, 0.5% (w/v) SDS, 5 × Denhardt's solution (10), and 100 µg/ml denatured salmon sperm DNA (Sigma Chem. Co.)) at 60 °C for 24-48 hours. For low stringency conditions filters were washed three times for thirty minutes with 500 ml of 3×SSC/0.5% (w/v) SDS at 60 °C. For high stringency conditions the washings were done with 0.1×SSC/0.5% (w/v) SDS at 68 °C. In cases where filters were probed more than once, the original probe was removed by a one hour wash at 70 °C in 1.0×SSC/50% (v/v) deionized formamide (using Bio-Rad Analytical Grade Mixed Bed Resin AG501-X8). Autoradiographic exposures were for 12 hours to 3 days at -80 °C using Kodak X-Omatic intensifying screens on Kodak XRP or XAR5 X-ray films.

2.8. *ILV3* probes used in Southern analyses

Three different *ILV3* probes were used during the course of this research: (i) The 3.6 kb BamHI fragment from plasmid pE30/5-6-3 (33), containing at least part of the *ILV3* gene of *S. cerevisiae* S288C, was used as a probe to screen hybrid plasmids containing *Saccharomyces carlsbergensis* inserts which complemented *ilv3* mutants of *Saccharomyces cerevisiae*. (ii) The 3.0 kb BamHI-EcoRI fragment from plasmid pGCT-16-1 (this study) known to contain a functionally complete allele of *ILV3* from *Saccharomyces carlsbergensis* strain 244 and which under high stringency washing conditions remains hybridized to the pE30/5-6-3 *ILV3* probe. (iii) The 2.9 kb AvaI-SalI fragment from plasmid pGCT-12-1 (this study), also containing a func-

tionally complete allele of *ILV3* from strain 244 but which only hybridizes to the pE30/5-6-3 *ILV3* under low stringency conditions.

3. RESULTS

3.1. Cloning of two alleles of *ILV3* from

Saccharomyces carlsbergensis strain 244

Saccharomyces cerevisiae IVPX5-2B (*ilv3-12*) was transformed with 10 µg of recombinant plasmid DNA from each of four pools as described in the Materials and Methods (2.5). Approximately 3,000-4,000 Ura⁺ transformants arose from each pool on plates of SC-ura. After replica plating to SC-ura-ile-val, 33 Ura⁺Ile⁺Val⁺ clones were selected at random from the four pools. Of these, 22 were unstable for the Ura⁺Ile⁺Val⁺ phenotype, while the others were stable Ura⁺Ile⁺Val⁺ (likely due to integration of the transforming plasmid into the yeast chromosome by sequence homology). Four of the unstable clones were selected and the complementing plasmids isolated. Restriction endonuclease site maps of the insertions in plasmids pGCT-16-1 and pGCT-22-1, obtained by single and double digestions, are shown in Figures 4a and 4b. The 5.3 kb insert in plasmid pGCT-16-1 has a physical map similar to a region in plasmid pGCT-22-1 (where the insert is 12.2 kb long), i.e. the insert in pGCT-16-1 is likely a smaller fragment of contiguous genomic DNA. This common region in the two plasmids has the same cleavage map as the region of plasmid pE30/5-6-3 known to contain the *ILV3* gene of *Saccharomyces cerevisiae* S288C (33). In fact, the restriction site map of the first 7.5 kb of the pGCT-22-1 insert (beginning from the BamHI junction point between the vector and inserts) is identical to a region of similar length in pE30/5-6-3 (33). This provides strong evidence that *Saccharomyces carlsbergensis* 244 contains an allele of *ILV3* very similar or identical to that found in *Saccharomyces cerevisiae* S288C.

In Figures 5a and 5b the two other plasmids

Figure 5. Restriction site maps of *ilv3* complementing plasmids pGCT-12-1, pGCT-21-1, and pGCT-8/11. Plasmid pGCT-8/11 is a BamHI-SalI subclone of pGCT-21-1. The thick line is vector DNA and the thin line in *Saccharomyces carlsbergensis* DNA. The dotted lines represent regions of the inserts with homology to the *S. cerevisiae* *ILV3* probe (section 3.2) and the dashed lines represent regions which by Southern analysis (section 3.4) are concluded to be contiguous genomic DNA.

Table II. Complementation pattern of *Saccharomyces cerevisiae* *ilv3-33* and *ilv3-53* mutants by *ilv3-12* complementing plasmids.

Plasmid	Strain	
	<i>S. cerevisiae</i> C80-1133 (<i>ilv3-33</i>)	<i>S. cerevisiae</i> C80-1153 (<i>ilv3-53</i>)
pGCT-12-1	+	+
pGCT-16-1	+	+
pGCT-21-1	+	+
pGCT-22-1	+	+

are depicted, pGCT-12-1 (containing a 9.1 kb insert) and pGCT-21-1 (containing a 12.8 kb insert). These have a completely different arrangement of cleavage sites as those mapped in Figures 4a and 4b. Plasmids pGCT-12-1 and pGCT-21-1 have a 2.9 kb region in common but differ in their cleavage maps outside of this common region.

3.2. Identification of the cloned genes as alleles of *ILV3*

Two methods were used to verify that the *ilv3* complementing plasmids contained an *ILV3* gene and thereby demonstrate that their effects were not due to genetic or physiological suppression of the *ilv3-12* mutation. In the first method, the four plasmids were used to transform strains C80-1133 and C80-1153 (Table II) representing two other alleles of *ilv3*. It was found that all four plasmids were able to complement the different mutant alleles.

In the second method the four plasmids were probed with a 3.6 kb BamHI fragment containing *ILV3* from *Saccharomyces cerevisiae*. Under low stringency conditions (i.e. 60 °C, 3×SSC) all

four plasmids had regions of homology with the *ILV3* probe, indicating that all contain an *ILV3* gene (Figure 6a). In addition, by using different combinations of restriction enzyme cleavage it was possible to localize the regions within the inserts which were homologous to the *ILV3* probe (Figures 4a, 4b, 5a, and 5b). As anticipated from their restriction site maps, the region of homology in plasmids pGCT-16-1 and pGCT-22-1 coincided with the region where the restriction site map was identical to that of the probe. This provides further evidence that this allele of *ILV3* from *Saccharomyces carlsbergensis* is very similar, if not identical, to the *ILV3* gene found in *Saccharomyces cerevisiae* S288C. In the case of plasmids pGCT-12-1 and pGCT-21-1 it was the region with the shared restriction site map which showed homology to the *ILV3* probe.

By increasing the stringency conditions of the filter washes to 60 °C and 0.1×SSC, the *ILV3* probe no longer hybridized to plasmids pGCT-12-1 and pGCT-21-1 (Figure 6b), whereas the hybridization intensity of the *ILV3* probe to plasmids pGCT-16-1 and pGCT-22-1 remained unaffected. Apparently, plasmids pGCT-12-1 and pGCT-21-1 contain an allele of *ILV3* with

Figure 6. Southern hybridization of restriction digests of pGCT-12-1, pGCT-16-1, pGCT-21-1, and pGCT-22-1 plasmid DNA (containing *S. carlsbergensis* genomic DNA) under (a) low stringency (60 °C, 3×SSC) and (b) high stringency (68 °C, 0.1×SSC) conditions. The probe was a 3.6 kb BamHI fragment from pE30/5-6-3 containing at least part of the *ILV3* gene of *S. cerevisiae*. Samples were: Lane A, HindIII-PvuII restricted pGCT-12-1. Lane B, EcoRI-PvuII cleaved pGCT-12-1. Lane C, SalI cleaved pGCT-12-1. Lane D, BamHI-XbaI cleaved pGCT-16-1. Lane E, XbaI cleaved pGCT-16-1. Lane F, HindIII-EcoRI cleaved pGCT-16-1. Lane G, BglII-BamHI cleaved pGCT-16-1. Lane H, BamHI cleaved pGCT-21-1. Lane I, BamHI-SalI cleaved pGCT-21-1. Lane J, EcoRI-SalI cleaved pGCT-21-1. Lane K, BamHI cleaved pGCT-22-1. Lane L, EcoRI cleaved pGCT-22-1. Lane M, HindIII cleaved pGCT-22-1. Lane N, λ -EcoRI. Lane O, λ -HindIII.

a) Low Stringency (60°C, 3XSSC)

b) High Stringency (68°C, 0.1XSSC)

only partial sequence homology to *ILV3* of *S. cerevisiae*, indicating it to be an allele of *ILV3* unique to *Saccharomyces carlsbergensis*.

3.3. Subcloning of the *ILV3* alleles

Using the data obtained from Southern analyses of the *ilv3* complementing plasmids, regions of plasmids pGCT-16-1 (containing an allele of *ILV3* apparently identical to that found in *Saccharomyces cerevisiae* S288C) and pGCT-21-1 (containing an allele of *ILV3* unique to *Saccharomyces carlsbergensis*) were subcloned into YRp17. As illustrated in Figures 4c and 5c, the 3.0 BamHI-EcoRI fragment of pGCT-16-1 (of which 0.375 kb is prokaryotic vector DNA) and the 3.4 kb BamHI-SalI insert fragment of pGCT-21-1 were cloned into BamHI-EcoRI and BamHI-SalI cleaved YRp17, respectively. The two subclones retained the ability to complement the *ilv3* mutation in *S. cerevisiae* IVPX5-2B indicating that the subcloned inserts contain fully functional alleles of *ILV3*. The previously smallest *S. cerevisiae* DNA fragment reported to complement the *ilv3* mutation in *Saccharomyces cerevisiae* was 4.5 kb (33).

3.4. Southern hybridization analyses with the two *ILV3* genes from *Saccharomyces carlsbergensis* as probes

Figures 7a and 7b illustrate the hybridization pattern obtained after probing total DNA from *Saccharomyces cerevisiae* and *Saccharomyces carlsbergensis* with DNA fragments harboring the two different *ILV3* genes from *Saccharomyces carlsbergensis*. The patterns demonstrate that the fragments used as probes correspond to unique DNA sequences in *S. carlsber-*

gensis and that there are two alleles of the *ILV3* gene.

Under low stringency conditions (i.e. 60 °C, 3×SSC) both probes hybridize to the same DNA fragments in all restriction digests (compare Figure 7a with 7b). HindIII cleavage of *Saccharomyces carlsbergensis* DNA yields hybridizing fragments of 7.8, 4.8, and 3.8 kb. The latter two are unique to the lager yeast genome (Figure 7a, lane A), and are not detected in *Saccharomyces cerevisiae* DNA by either probe (Figures 7a, 7b, lane D). The 7.5 kb band is common to both strains, hybridizing with the same intensity to the pGCT-16-1 probe containing the *Saccharomyces cerevisiae* like *ILV3* allele. Hybridization is not detected at 68 °C/0.1×SSC when the pGCT-12-1 *ILV3* probe is used (containing the allele of *ILV3* unique to the lager yeast). The HindIII hybridization patterns also confirm that the largest HindIII-HindIII fragment in the insert in pGCT-22-1 represents contiguous genomic DNA from *Saccharomyces carlsbergensis* 244, as a fragment of this size is predicted from the restriction site map.

The BglII-SalI cleavage of *Saccharomyces carlsbergensis* DNA yields fragments of 6.8, 3.9 (a doublet), and 1.6 kb (Figure 7, lane B). By comparing the hybridization patterns seen at 60 °C and 68 °C with the two probes it can be seen that the 6.8 kb fragment is also found in *Saccharomyces cerevisiae* M1153 (Figure 7, lane E), but with the pGCT-12-1 probe it is only detected under low stringency conditions. The 3.9 doublet consists of a fragment found in *Saccharomyces cerevisiae* (confirming that this fragment in the pGCT-22-1 insert represents contiguous genomic DNA) and of a fragment unique to the *Saccharomyces carlsbergensis* genome (confirming that this fragment in the

---

Figure 7. Southern hybridization analysis of *Saccharomyces carlsbergensis* 244 and *Saccharomyces cerevisiae* C80-1153 DNA under low (60 °C, 3×SSC) and high (68 °C, 0.1×SSC) stringency conditions when probed with the 2.9 kb Aval-SalI fragment of pGCT-12-1 containing the *ILV3* allele of *S. carlsbergensis* unique to this strain (Figure 7a) and the 3.0 kb BamHI-EcoRI fragment of pGCT-16-1 containing the *S. cerevisiae* like *ILV3* of *S. carlsbergensis* (Figure 7b). The same filter was used for both probes and the following restriction endonuclease digestions were carried out: Lanes A and D, HindIII cleaved *S. carlsbergensis* and *S. cerevisiae* DNA, respectively. Lanes B and E, BglII-SalI cleaved *S. carlsbergensis* and *S. cerevisiae* DNA, respectively. Lanes C and F, XbaI-SalI cleaved *S. carlsbergensis* and *S. cerevisiae* DNA, respectively. Lanes G, H, and I, EcoRI, HindIII, and EcoRI-HindIII cleaved λ -DNA, respectively.

a)

pGCT-12-1 *ILV3* probe

Low Stringency (60°C, 3XSSC)

A B C D E F G H I

High Stringency (68°C, 0.1XSSC)

A B C D E F G H I

b)

pGCT-16-1 *ILV3* probe

Low stringency (60°C, 3XSSC)

A B C D E F G H I

High Stringency (68°C, 0.1XSSC)

A B C D E F G H I

Figure 8. Southern hybridization analysis of *Saccharomyces carlsbergensis* DNA under (a) low (60 °C, 3×SSC) and (b) high (68 °C, 0.1×SSC) stringency conditions when probed with the *ILV3* allele of pGCT-12-1. Lane A, PvuI cleaved *S. carlsbergensis* DNA. Lane B, λ-EcoRI. Lane C, λ-HindIII.

pGCT-21-1 insert represents contiguous genomic DNA). The 1.6 kb fragment is unique to the lager's yeast genotype and confirms that the 1.6 kb BglII-SalI fragment within the probe itself represents contiguous genomic DNA.

In the XbaI-SalI double cleavage (Figure 7, lanes C and F) 9.5, 3.4 (doublet), and 2.6 kb DNA fragments in the lager yeast hybridize to the *ILV3* probes under low stringency conditions. The 9.5 kb fragment is unique to the 244 lager yeast while the 2.6 kb and one of the 3.4 fragments are common to both yeast genotypes. The fact that an XbaI site is not found 9.5 kb away from the insert in pGCT-21-1 indicates

that the entire insert is not contiguous genomic DNA. However, the detection of a 9.3 kb fragment in the PvuI-SalI double cleavage (Figure 5c) indicates that at least this portion of the insert is contiguous DNA, with the ligation point of two pieces of genomic DNA being very near to the PvuI site.

The detection of 3.4 (doublet) and 2.6 kb XbaI-SalI fragments in both yeasts confirms that the DNA between the XbaI sites in the inserts of pGCT-16-1 (3.4 kb) and pGCT-22-1 (3.4 kb and 2.6 kb) represent contiguous genomic DNA. The lack of a 4.0 kb hybridizing fragment (XbaI-SalI) indicates that the restriction site

map of plasmid pGCT-12-1 does not represent contiguous genomic DNA outside of the region coding for the *ILV3* gene. Under low stringency conditions a 2.9 kb *Xba*I-*Sal*I fragment hybridizes to the pGCT-12-1 *ILV3* probe in both yeasts (but not the pGCT-16-1 probe). Such a fragment is not predicted on the basis of the *ILV3* restriction site maps of these plasmids and a satisfactory explanation cannot be given. The degree of sequence homology, however, appears to be very low as the signal is very weak.

4. DISCUSSION

This paper reports on the cloning of two alleles of a gene from a genomic library of a *Saccharomyces carlsbergensis* lager yeast. The two alleles of *ILV3*, coding for dihydroxyacid dehydrogenase, were cloned from *S. carlsbergensis* 244, a production lager strain of the Carlsberg Breweries. The presence of two alleles is supported by: 1. The cloned genes complement three different *ilv3* mutations in *S. cerevisiae*. 2. Both alleles, which have unique restriction site maps, hybridize in Southern analyses to an *ILV3* probe from *S. cerevisiae* S288C. 3. Southern type hybridization analyses of genomic DNA from *S. carlsbergensis* 244 reveal the presence of two alleles of *ILV3*, both of which can be accounted for with the knowledge of the restriction site maps of the inserts containing the cloned genes. In a subsequent paper (7) it is shown that the two alleles originate from different forms of chromosome X.

One of the *ILV3* alleles from the lager yeast is very similar or identical to the *ILV3* allele of *S. cerevisiae* S288C, since it is located in a chromosome segment with a restriction site map identical to that found in *S. cerevisiae* S288C over a 7.5 kb section of genomic DNA. In addition, in Southern analyses with the *ILV3* gene of *S. cerevisiae* S288C as a probe, this allele remains hybridized even under washing conditions giving high stringency.

The second allele of *ILV3* is clearly unique to the lager yeast genome. It has a restriction site map unrelated to that of the *ILV3* gene from *S. cerevisiae* (and its counterpart in the lager yeast) and does not hybridize to the *ILV3* of *S. cerevisiae* under high stringency washing conditions. There must, however, still be considerable

sequence homology between the domains containing the two alleles as hybridization is detected under low stringency washing conditions. As both alleles complement *ilv3* mutations in *S. cerevisiae* and have some sequence homology, the lager yeast most likely contains two dihydroxyacid dehydrogenase isoenzymes with somewhat different primary structure.

The presence of two alleles of *ILV3* in *S. carlsbergensis* indicates that the chromosomes on which these alleles are located, chromosome X, are different from each other at the nucleotide level and are not homologous copies. This observation is consistent with data obtained in studies with the same yeast on chromosomes III, V, VII, and XIII where two versions of these chromosomes, differing at the nucleotide level, have been reported (15, 27, 39). More direct evidence for this being the case with chromosome X will be presented in a later publication (7). The results presented here with *ILV3* are also consistent with reports on gene polymorphism between *S. cerevisiae* and *S. carlsbergensis* in the *CYC7*, *HIS4*, *ILV1*, *LEU2*, *MAT*, and *URA3* loci (15, 27, 28, 29, 30).

From a genetic engineering viewpoint the results obtained here with *ILV3* have practical implications for research programs aimed at engineering a brewer's yeast which produces little or no diacetyl. For example, assuming that all of the enzymes of the isoleucine-valine pathways are encoded for by two alleles, strategies based on the insertional inactivation of structural *ILV* genes would require that both alleles of a particular gene be inactivated. Likewise, strategies based on increasing the copy number of *ILV* genes in lager yeast can benefit from the possibility to select from two alternative alleles of each gene. To assist in such programs, the sequencing of the two alleles of *ILV3* in *S. carlsbergensis* 244 is presently in progress. Such information on the sequence homology among the two alleles will also be useful for tracing the evolution of lager yeast genomes within the genus *Saccharomyces*.

ACKNOWLEDGEMENTS

I wish to express my gratitude to the following persons for their contribution to this work.

CLAES GJERMANSSEN, STEEN HOLMBERG, MORTEN KIELLAND-BRANDT, JENS G. LITSKE PETERSEN, JULIO POLAINA, TORSTEN NILSSON-TILLGREN, and DITER VON WETTSTEIN I thank for useful discussions throughout the course of the research. MORTEN KIELLAND-BRANDT and DITER VON WETTSTEIN for critical reading of the manuscript. The donation of plasmid pE30/5-6-3 by JULIO POLAINA is gratefully acknowledged. GITTE BANK and KIRSTEN TERKILDSEN have provided technical assistance. ANN-SOFI STEINHOLTZ and NINA RASMUSSEN have prepared the figures. The author was supported by a Canadian government NSERC postdoctoral fellowship.

REFERENCES

- ANDERSON, E. & P. A. MARTIN: The sporulation and mating of brewing yeasts. *J. Inst. Brew.* 81, 242-247 (1975)
- BIRNBOIM, H. C. & J. DOLY: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* 7, 1513-1523 (1979)
- BOTSTEIN, D. & R. W. DAVIS: Principles and practice of recombinant DNA research with yeast. In: *Molecular Biology of the Yeast Saccharomyces. Metabolism and Gene Expression*. J. N. Strathern, E. W. Jones & J. R. Broach eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, vol 11B, pp. 607-636 (1982)
- BOYER, H. W. & D. ROULLAND-DUSSOIX: A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41, 459-472 (1969)
- BUSSEU, H. & H. E. UMBARGER: Biosynthesis of branched-chain amino acids in yeast: Regulation of synthesis of the enzyme of isoleucine and valine biosynthesis. *J. Bacteriol.* 98, 623-628 (1969)
- CABANE, B., C. RAMOS-JEUNEHOMME, N. LAPAGE & C. A. MASSCHELEIN: Vicinal diketones - the problem and prospective solutions. *Amer. Soc. Brew. Chem. Proc.* 1973. pp. 94-99 (1974)
- CASEY, G. P.: Molecular and genetic analysis of chromosomes X in *Saccharomyces carlsbergensis*. *Carlsberg Res. Commun.* 51, 343-362 (1986)
- DAVIS, R. W., H. THOMAS, J. CAMERON, T. P. ST JOHN, S. SCHERER & R. A. PADGETT: Rapid DNA isolations for enzymatic and hybridization analysis. *Methods in Enzymology*, vol. 65, Part I, pp. 404-414 (1980)
- DELFORGE, J., F. MESSENGUY & J. WIAME: The regulation of arginine biosynthesis in *Saccharomyces cerevisiae*: The specificity of *argR* mutations and the general control of amino acid biosynthesis. *Europ. J. Biochem.* 57, 231-239 (1975)
- DENHARDT, D. T.: A membrane-filter technique for the detection of complementary DNA. *Biochem. Biophys. Res. Comm.* 23, 641-646 (1966)
- FALCO, S. C., K. S. DUMAS & K. J. LIVAK: Nucleotide sequence of the yeast *ILV2* gene which encodes acetolactate synthase. *Nucleic Acids Res.* 13, 4011-4027 (1985)
- FALCO, S. C. & K. S. DUMAS: Genetic analysis of mutants of *Saccharomyces cerevisiae* resistant to the herbicide sulfmeturon methyl. *Genetics* 109, 21-35 (1985)
- FOWELL, R. R.: Sporulation and hybridization of yeasts. In: *The Yeasts*. A. H. Rose & J. S. Harrison eds. Academic press, London, vol. 1, pp. 303-383 (1969)
- FREUNDLICH, M., R. BURNS & H. UMBARGER: Control of isoleucine, valine, and leucine biosynthesis. I. Multivalent repression. *Proc. Natl. Acad. Sci. USA* 48, 1804-1808 (1962)
- HOLMBERG, S.: Genetic differences between *Saccharomyces carlsbergensis* and *S. cerevisiae* II. Restriction endonuclease analysis of genes in chromosome III. *Carlsberg Res. Commun.* 47, 233-244 (1982)
- HOLMBERG, S., M. KIELLAND-BRANDT, T. NILSSON-TILLGREN & J. G. L. PETERSEN: The *ILV1* gene in *Saccharomyces cerevisiae*: 5' and 3' end mapping of transcripts and their regulation. *Carlsberg Res. Commun.* 50, 163-178 (1985)
- ITO, H., Y. FUKUDA, K. MURATA & A. KIMURA: Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* 153, 163-168 (1983)
- JOHNSTON, J. R.: Breeding yeasts for brewing, I. Isolation of breeding strains. *J. Inst. Brew.* 71, 130-135 (1965)
- KAKAR, S. N. & R. P. WAGNER: Genetic and biochemical analysis of isoleucine-valine mutants of yeasts. *Genetics* 49, 213-222 (1964)
- KIELLAND-BRANDT, M. C., S. HOLMBERG, J. G. L. PETERSEN & T. NILSSON-TILLGREN: Nucleotide sequence of the gene for threonine deaminase (*ILV1*) of *Saccharomyces cerevisiae*. *Carlsberg Res. Commun.* 49, 567-575 (1984)
- KIELLAND-BRANDT, M. C., T. NILSSON-TILLGREN, S. HOLMBERG, J. G. L. PETERSEN & B. A. SVENNINGSEN: Transformation of yeast without the use of foreign DNA. *Carlsberg Res. Commun.* 44, 77-87 (1979)
- MAGEE, P. T. & L. M. HEREFORD: Multivalent repression of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. *J. Bacteriol.* 98, 857-862 (1969)

23. MAGEE, P. T. & H. DE ROBICHON-SZULMAJSTER: The regulation of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. 2. Identification and characterization of mutants lacking the acetohydroxy-acid synthetase. *Eur. J. Biochem.* 3, 502-506 (1968)
24. MANDEL, M. & A. HIGA: Calcium-dependent bacteriophage DNA infection. *J. Mol. Biol.* 53, 159-162 (1970)
25. MANIATIS, T., E. F. FRITSCH & J. SAMBROOK: *Molecular Cloning. A Laboratory Manual*. Cold Harbor Laboratory, Cold Spring Harbor, New York (1982)
26. MORTIMER, R. K. & D. SCHILD: Genetic map of *Saccharomyces cerevisiae*, Edition 9. *Microbiol. Rev.* 49, 181-212 (1985)
27. NILSSON-TILLGREN, T., C. GJERMENSEN, M. C. KIELLAND-BRANDT, J. G. L. PETERSEN & S. HOLMBERG: Genetic differences between *Saccharomyces carlsbergensis* and *S. cerevisiae*. Analysis of chromosome III by single chromosome transfer. *Carlsberg Res. Commun.* 46, 65-76 (1981)
28. NILSSON-TILLGREN, T., M. C. KIELLAND-BRANDT, S. HOLMBERG, J. G. L. PETERSEN & C. GJERMENSEN: Is lager yeast a species hybrid? Utilization of intrinsic genetic variation in breeding. *Proc. IV, Internat. Symp. Genet. Industr. Microorg.* Kyoto, pp. 143-147 (1982)
29. PEDERSEN, M. B.: DNA sequence polymorphisms in the genus *Saccharomyces*. I. Comparison of the *HIS4* and ribosomal RNA genes in lager strains, ale strains and various species. *Carlsberg Res. Commun.* 48, 485-503 (1983)
30. PEDERSEN, M. B.: DNA sequence polymorphisms in the genus *Saccharomyces*. II. Analysis of the genes *RDN1*, *HIS4*, *LEU2* and *Ty* transposable elements in Carlsberg, Tuborg and 22 Bavarian brewing strains. *Carlsberg Res. Commun.* 50, 263-272 (1985)
31. PETERSEN, J. G. L., S. HOLMBERG, T. NILSSON-TILLGREN & M. C. KIELLAND-BRANDT: Molecular cloning and characterization of the threonine deaminase (*ILV1*) gene of *Saccharomyces cerevisiae*. *Carlsberg Res. Commun.* 48, 149-159 (1983)
32. PETERSEN, J. G. L., M. C. KIELLAND-BRANDT, S. HOLMBERG & T. NILSSON-TILLGREN: Mutational analysis of isoleucine-valine biosynthesis in *Saccharomyces cerevisiae*. Mapping of *ilv2* and *ilv5*. *Carlsberg Res. Commun.* 48, 21-34 (1983)
33. POLAINA, J.: Cloning of the *ILV2*, *ILV3* and *ILV5* genes of *Saccharomyces cerevisiae*. *Carlsberg Res. Commun.* 49, 577-584 (1984)
34. RIGBY, P. W. J., M. DIECKMANN, C. RHODES & P. BERG: Labelling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. *J. Mol. Biol.* 113, 237-251 (1977)
35. RYAN, E. D. & G. B. KOHLHAW: Subcellular localization of isoleucine-valine biosynthetic enzymes in yeast. *J. Bacteriol.* 120, 631-637 (1974)
36. SATYANARAYANA, T., H. E. UMBARGER & G. LINDEGREN: Biosynthesis of branched-chain amino acids in yeast: Correlation of biochemical blocks and genetic lesions in leucine auxotrophs. *J. Bacteriol.* 96, 2012-2017 (1968)
37. SOUTHERN, E. M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98, 503-517 (1975)
38. THORNE, R. S. W.: The genetics of flocculence in *Saccharomyces cerevisiae*. *Compt. Rend. Lab. Carlsberg, Ser. Physiol.* 25, 101-140 (1951)
39. WETTSTEIN, D. VON, C. GJERMENSEN, S. HOLMBERG, M. C. KIELLAND-BRANDT, T. NILSSON-TILLGREN, M. B. PEDERSEN, J. G. L. PETERSEN & P. SIGSGAARD: Genetic engineering in the improvement of brewers yeast. *MBAA Tech. Quart.* 21, 45-61 (1984)
40. WINGE, O.: On segregation and mutation in yeast. *Compt. Rend. Lab. Carlsberg, Ser. Physiol.* 24, 79-96 (1944)
41. YANG, R. C. A., J. LIS & R. WU: Elution of DNA from agarose gels after electrophoresis. In: *Methods in Enzymology*, R. Wu, ed. Academic Press, New York, vol. 68, pp. 179-182 (1979)

Accepted by H. KLENOW