DNA SEQUENCE POLYMORPHISMS IN THE GENUS SACCHAROMYCES. II. ANALYSIS OF THE GENES *RDN1*, *HIS4*, *LEU2* AND *Ty1* TRANSPOSABLE ELEMENTS IN CARLSBERG, TUBORG AND 22 BAVARIAN BREWING STRAINS

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The genotypes of the Carlsberg and Tuborg bottom fermenting (lager) *Saccharomyces* strains as well as those of 13 other bottom fermenting yeasts and 9 top fermenting yeasts from the collection of the Wissenschaftliche Station für Brauerei in Munich were analysed for restriction fragment length differences in the *RDN1* ribosomal RNA gene cluster on chromosome XII as well as in the chromosome III regions containing the *HIS4* (histidine 4) and *LEU2* (leucine 2) genes. All brewing strains had the same form II *RDN1* gene cluster. The top fermenting strains yielded identical *Sall* restriction endonuclease fragment patterns (I) when probed with the *HIS4* gene probe of plasmid pC503. The pattern corresponds to that found in genetic standard strain S288C. The bottom fermenting strains were all heterozygous for the *HIS4* patterns I and II known to originate from homeologous regions of two chromosomes.

Molecular hybridisation of a *LEU2* gene probe to *Sall* restricted DNA of the bottom fermenting yeast strains gave identical fragment patterns (III + IV), while a pattern designated II + III was most common in top fermenting strains. Two additional patterns were found among the latter.

The transposable element *Ty1* from a genetic standard strain distinguishes the genotypes of various lager strains as well as those of top fermenting yeasts. Hybridisation of *Ty1* to *EcoRI* restricted DNA of Carlsberg lager strain BK 2246 yields four diagnostic fragments, while the DNA of Tuborg lager strain BK 2224 is characterized by an additional 5.5 kb fragment. Conversely *Sall* restricted DNA of the Carlsberg strain contains a 10 kb fragment absent from the genome of the Tuborg strain. Three different restriction fragment patterns are found after probing with the *Ty1* element among the Bavarian bottom fermenting yeasts. More complex patterns with at least 10 restriction fragments hybridising to the *Ty1* element are found among the top fermenting strains from the Munich collection. Five different genotypes have been identified among 9 strains tested. *Ty1* transposing elements will be helpful markers in the characterisation of improved brewing strains.

1. INTRODUCTION

Restriction endonuclease fragment analyses by molecular hybridisation with radioactively labelled, cloned gene probes are useful for monitoring gene polymorphisms in *Saccharomyces*

strains (15, 18, 19, 20, 21, 22, 23). In a previous paper, top fermenting ale strains could be distinguished from bottom fermenting (lager) strains by polymorphisms at the *HIS4* and the *RDN1* loci (20), but only the Bavarian Pschorr

Abbreviations: SSC = 0.15 M-NaCl, 15 mM-Na citrate; TBE = 90 mM-Tris base, 90 mM-boric acid, 2 mM-EDTA, pH 8.3; TE = 10 mM-Tris HCl, 1 mM-EDTA, pH 7.5.

strain could be separated from other bottom fermenting strains. This particular strain is homozygous for a certain *HIS4* allele, designated as restriction fragment pattern II while the other strains are heterozygous and contain patterns I and II (18, 20).

In the present study 22 Bavarian brewing strains from the collection of the Wissenschaftliche Station für Brauerei Munich have been analysed at the *RDNI*, *HIS4*, *LEU2* and *Ty* regions and compared with the Carlsberg and Tuborg bottom fermenting strains. The *RDNI* locus on chromosome XII is a cluster of about 100 genes (25), each encoding a set of the 18S, 5.8S, 25S and 5S cytosolic ribosomal RNA molecules (24). Three forms of this gene have been detected in brewing and other *Saccharomyces* strains. Forms I and II yield upon cutting with *Hind*III endonuclease two fragments of 6.4 kb and 2.6 kb (24), while the latter fragment in form III contains an additional restriction site and form III thus yields three fragments with sizes of 6.4 kb, 1.45 kb and 1.15 kb (20). Forms I and II are distinguishable by their *Eco*RI restriction endonuclease patterns. Form I is cleaved into seven fragments (2.8 kb, 2.4 kb, 1.95 kb, 0.66 kb, 0.58 kb, 0.34 kb and 0.22 kb) and form II into only six fragments, since in this form the restriction site separating the 2.4 and 0.58 kb fragments is absent (24). The resulting *Eco*RI fragment of 3 kb of form II has in form III a reduced size of 2.8-2.9 kb, thus giving rise to a doublet in the pattern (20). Forms I or II are seen in *S. cerevisiae* strains, while all bottom fermenting strains investigated had form II. Form III has been found in *S. bayanus*, type strains of *S. carlsbergensis*, *S. monacensis* and *S. uvarum* (19, 20, 21, 23).

The *HIS4* gene is located on chromosome III of *S. cerevisiae* (Figure 1) and encodes three activities in the histidine biosynthesis. It occurs in the genus in at least three allelic forms which are recognisable by the restriction fragment patterns I, II (12, 18) and III (20). In the present study the three alleles are distinguished by their *Sal*I restriction endonuclease fragment patterns. Pattern I is characterised by fragments of the sizes 13.0 kb, 9.4 kb and 1.5 kb, while pattern II gives fragment sizes of 6.7 and 2.3 kb (12, 18). Pattern III has four fragments with sizes of 6.4

kb, 5.5 kb, 2.1 kb and 1.6 kb (20). The studies (19, 20, 21, 22, 23) have shown that the pattern I allele is present in nearly all brewing strains, while pattern II alone or together with I is seen in lager brewing strains in an *S. carlsbergensis* type strain and in *S. monacensis*. The pattern III allele is present in *S. uvarum* strains in homozygous condition or in *S. bayanus* strains together with the pattern II allele of *HIS4*.

The *LEU2* gene encoding the β -isopropylmalate dehydrogenase (2) is like the *HIS4* gene located on chromosome III (Figure 1) in genetic type strains of *S. cerevisiae* (4). Several alleles of *LEU2* have been detected in distillers yeast and other *Saccharomyces* strains (15, 21, 22, 23). At least five *LEU2* restriction fragment patterns occur in top and bottom fermenting strains (21, 22).

The *Ty* elements resemble retroviruses in structure and function (3, 7, 8, 9) and are found to be distributed at several positions in the *S. cerevisiae* genome (16). Polymorphisms for *Ty* elements among brewing strains were investigated with the p14 probe corresponding to the central portion of the *TyI* element (Figure 1) (26) rather than a full *Ty* element. This was done in order to avoid cross-hybridisation to the 338 bp long terminally repeated δ -sequences. These terminal repeats present at many sites in the genome as remnants after excision of *Ty* elements.

2. MATERIALS AND METHODS

2.1. Strains and media

The 13 Bavarian bottom fermenting strains are listed together with the Carlsberg and Tuborg strains in Table I while the 9 Bavarian top fermenting strains are listed in Table II. Complete media plates (YPD) were made up according to SHERMAN, FINK and HICKS (28).

2.2. Preparation of DNA

Yeast DNA was isolated essentially as in (20). Cells were scraped from a YPD plate which previously had been incubated for 48 hours, suspended in 5 ml 1 M-sorbitol, spun down at 4000 g and resuspended in sorbitol containing 20 mM-dithiothreitol (Sigma D-0632 USA) and

Table I.

Characteristics of the analysed regions of the bottom fermenting strains (*Saccharomyces carlsbergensis*). WSB No. = Wissenschaftliche Station für Brauerei, Munich culture number.

Strain	<i>RDN1</i> form	<i>HIS4</i> pattern	<i>LEU2</i> fragments	<i>Ty</i> pattern	Type	WSB No.
C83-FP34	II	I+II	III+IV	IIb	Untergärige Bruchhefe	(149)
C83-FP35	II	I+II	III+IV	I	do.	(307)
C83-FP36	II	I+II	III+IV	IIa	do.	(308)
C83-FP37	II	I+II	III+IV	IIb	Untergärige Staubhefe	(310)
C83-FP38	II	I+II	III+IV	IIa	Untergärige Bruchhefe	(314)
C83-FP39	II	I+II	III+IV	IIa	do.	(315)
C83-FP40	II	I+II	III+IV	IIa	do.	(316)
C83-FP41	II	I+II	III+IV	IIa	do.	(319)
C83-FP42	II	I+II	III+IV	IIa	do.	(320)
C83-FP43	II	I+II	III+IV	IIa	do.	(328)
C83-FP44	II	I+II	III+IV	IIa	do.	(329)
C83-FP46	II	I+II	III+IV	IIa	do.	(334)
C83-FP47	II	I+II	III+IV	IIa	do.	(344)
Carlsberg lager BK2246	II	I+II	III+IV	I		
Tuborg lager BK 2224	II	I+II	III+IV	II		

incubated at 30 °C for 30-45 min. Then zymolase-60000 (from *Arthrobacter luteus*, Seikagaku Kogyo Co. Ltd., Japan) was added (1.4 mg). The solution was further incubated at 30 °C for 30 min. Cell lysis was induced by addition of 0.2 ml 10% sodium dodecyl sulfate (SDS), 0.5 ml of 0.5 M-EDTA (pH 8.5) and 0.5 ml 2 M-Tris base (tris-(hydroxymethyl)-amino methane). After stirring the solution was heated to 65 °C for 20 min. The tube was then cooled on ice and one

ml of 5 M-potassium acetate was added. After one hour on ice the tube was spun for 10-15 min at 4000 g and the nucleic acids in the supernatant were precipitated with 1 volume of 2-propanol:water, 5:1 (v/v). After one hour at -20 °C the precipitate was spun down at 4000 g for 10 min, resuspended in 10 mM-Tris, 1 mM-EDTA buffer (pH 7.5) and 2.5 µg ribonuclease A (Sigma R-5125) were added. The ribonuclease treatment was performed for 30 min at room tempe-

Table II.

Characteristics of top fermenting strains (*Saccharomyces cerevisiae*). WSB No. = Wissenschaftliche Station für Brauerei, Munich culture number.

Strain	<i>RDN1</i> form	<i>HIS4</i> pattern	<i>LEU2</i> fragments	<i>Tyn</i> pattern	Type	WSB No.
C83-FP48	II	I	7kb+III	- FP48	Obergärige Weissbierhefe	(297)
C83-FP49	II	I	II+III	- FP49	do.	(303)
C83-FP50	II	I	II+III	- FP50	Obergärige Alehefe	(306)
C83-FP51	II	I	II+III	- FP49	Obergärige Weissbierhefe	(317)
C83-FP52	II	I	II+III	- FP52	Obergärige Altbierhefe	(318)
C83-FP53	II	I	II+III	- FP49	Obergärige Weissbierhefe	(332)
C83-FP54	II	I	II+III	- FP49	do.	(333)
C83-FP55	II	I	5kb+III	- FP55	do.	(335)
C83-FP56	II	I	II+III	- FP49	do.	(340)

Figure 1. Restriction maps of the probes used in this study.

A: Restriction map of the transposable element *Ty1* in *Saccharomyces cerevisiae* according to PHILIPPSEN et al. (26). The 1.5 kb *Ty1* probe is also referred to as the *Ty1* "right" probe. (Hatched area). Filled areas correspond to the δ -sequences of 338 bp.

B: A partial genetic map of chromosome III in *S. cerevisiae* with expanded *LEU2* and *HIS4* restriction maps according to (4, 6, 11). The probes used in this study are indicated. The plasmid pC503 contains the 9.4 kb *PstI* insert shown and the arrow marks the *HIS4* gene. The insert of plasmid pC512 consists of the 2.4 kb *SalI* fragment which includes the entire *LEU2* gene, an adjacent tRNA gene and part of a *Ty1-17* element (1, 6, 10, cf 15). The 0.9 kb probe used contains the centromere proximal part of the *LEU2* gene. Abbreviation for restriction sites: H = HindIII, P = *PstI*, R = *EcoRI*, S = *SalI*.

perature. The DNA was precipitated at -20°C over night by the addition of 2-propanol:water and 1/10 volume of saturated sodium acetate. The DNA was spun down in the Eppendorf centrifuge and resuspended in TE buffer, 100-200 μl depending on the yield of the specific strain.

Plasmid DNA from *E. coli* was isolated in preparative scale according to HOLMES and QUIGLEY (13).

2.3. Molecular hybridisation analyses

Restriction endonucleases were obtained from Boehringer Mannheim and used according to MANIATIS, FRITSCH and SAMBROOK (17). DNA fragments were separated by gel electrophoresis in 0.7% agarose. The gels were stained with ethidium bromide at a concentration of 1 $\mu\text{g}/\text{ml}$ in $1 \times \text{TBE}$ buffer. DNA fragments were transferred to nitrocellulose (Millipore HAWP

I	II	I&II	III	II&III	kb					Fragment
—		—			13.0	=	—	—	—	III
—		—			9.4			—		IV
	—	—	—	=	6.7		—			II
			—	—	5.5				—	7 kb
										5 kb
	—	—	—	=	2.3					
					2.1					
—		—	—	—	1.6					
					1.5					
HIS4						LEU2				

Figure 2. Diagnostic key to the *HIS4* and *LEU2* Sall restriction fragment patterns. Fragment sizes are in thousands of nucleotide pairs (kb).

00010 or Schleicher and Schuell BA 85) in $20 \times$ SSC (14, 29). Labelling of the probes with [α - 32 P]dATP (NEG-012A New England Nuclear) was performed by nick translation according to RIGBY et al. (27) using DNA polymerase I (Boehringer Mannheim No. 104 493) at 14 °C for 75-90 minutes. Molecular hybridisation of labelled probes to filter bound DNA was done in $3 \times$ SSC, $10 \times$ Denhardt's solution (5, 17) at 60 °C for 18-24 hours. Filters were rinsed in $1 \times$ SSC + 0.2% SDS at room temperature for 2×10 min and 1×10 min at 60 °C. Autoradiograph was done at -80 °C with Kodak X-Omatic screens on Kodak XRP or XAR5 X-ray films for 15 h to 12 days.

2.4. Probes (cf. Figure 1)

The plasmid pC503 containing the 9.4 kb PstI fragment covering the *HIS4* gene from *S. cerevisiae* (11) was used as *HIS4* probe. The 931 bp EcoRI-SalI fragment covering the 3' end of the *LEU2* coding region from *S. cerevisiae* (10) was used as *LEU2* probe. This 0.9 kb fragment originates from a plasmid (pC512), (HOLMBERG, unpubl.) which consists of the vector

pBR325 containing a 2.33 kb Sall insert covering the *LEU2* gene from *S. cerevisiae* strain S288C. The Sall insert covers besides the entire *LEU2* gene a tRNA and part of the *Ty* element *Ty1-17* (1, 4, 6, 12, 30). The 0.9 kb EcoRI-SalI fragment is used as probe to avoid cross hybridisation to tRNAs and δ -sequences (12, 15). The *Ty1* probe was the 1.5 kb EcoRI-SalI fragment of *S. cerevisiae Ty1*, hatched in Figure 1A. A plasmid p14, consisting of this fragment in the vector pBR322 was kindly donated by P. PHILIPPSEN. The 0.9 kb *LEU2* probe was prepared from a digest of pC512 by electroelution onto a dialysis membrane (33).

3. RESULTS

3.1. Characterisation of the *RDNI* region

Due to the amplification of the *RDNI* gene it is possible to identify the *RDNI* forms directly on the ethidium bromide stained gels in UV light. The characterization of the *RDNI* forms are here based on the EcoRI digested DNA (20, 24). The analysed brewing strains all contain the *RDNI* form II (Tables I and II) in agreement

Figure 3. *Sall* restriction endonuclease fragment patterns of *LEU2* genes in the Bavarian brewing strains. Lane a: bottom fermenting strain C83-FP47, lane b, c and d: top fermenting strains C83-FP48, C83-FP50 and C83-FP55. Fragment sizes are in thousand of nucleotide pairs (kb). Molecular hybridisation of the probe was performed in 3×SSC at 60 °C and the filters were washed in 1×SSC at 20 °C for 2×10 min.

with the exclusive presence of this *RDN1* allele in other continental European brewing strains (19, 20, 21).

3.2. Molecular hybridisation with the *HIS4* probe pC503 from *S. cerevisiae*

Surveying of the *HIS4* genes was done on *Sall* digested DNA preparations, since previous analyses (20) have revealed the *Sall* fragment size patterns of the three known alleles to be easily distinguishable (Figure 2). The analysed bottom

fermenting strains contained the summation of combination *HIS4* patterns I and II (Table I). In contrast, the top fermenting strains all contained pattern I alone (Table II). While there is to date no bottom fermenting strain known which only contains the pattern I *HIS4* allele, two lager brewing strains of production quality have been identified which are hemi- or homozygous for the *HIS4* pattern II. One is the strain used formerly by the Bavarian Brewery Pschorr (20), and the other is the new hybrid strain C80-CG133 (31, 32).

3.3. Molecular analyses of the *LEU2* locus in brewing strains

The DNA preparations studied with the *LEU2* probe have been digested with the *Sall* restriction endonuclease. Four different hybridisation patterns have been observed (Figures 2 and 3). The first pattern (Figure 3a) is characterised by hybridisation of the *LEU2* probe to *Sall* fragments III and IV with approximate sizes of 16 and 12 kb. This pattern is diagnostic for all 15 lager brewing strains. The other three patterns are found among the top fermenting strains and are easily recognised by the absence of the 12 kb fragment IV. Seven of the nine top fermenting yeasts had the pattern shown in Figure 3c which consists of an about 16 kb fragment (III) and a fragment (II) of approximately 10 kb. The two strains C83-FP48 and C83-FP55 differ by either containing a 7 kb fragment (Figure 3b) or a 5 kb fragment (Figure 3d) together with fragment III.

3.4. Molecular analyses of regions in brewing strains that hybridise to the *Ty1* element from *S. cerevisiae*

When the radioactively labelled central portion of the *Ty1* element is hybridised to electrophoretically separated restriction endonuclease

Figure 4. Restriction endonuclease fragment patterns of *Ty* elements in brewing strains. A: The strains Carlsberg BK 2246 and Tuborg BK 2224 contain pattern I and II, respectively. B: The Bavarian strains C83-FP34, C83-FP35 and C83-FP36 represent the patterns II_b, I and II_a. C: *EcoRI* digests of the top fermenting strains C83-FP48, C83-FP49, C83-FP50, C83-FP52 and C83-FP55. Each strain represents a distinct fragment pattern. Fragment sizes in thousands of nucleotide pairs (kb). Hybridisation of the probe to filter bound DNA was performed in 3×SSC at 60 °C and the filters were washed in 1×SSC at 20 °C for 2×10 min. and 1×10 min at 60 °C.

fragments of the DNA from lager and top fermenting strains, extensive polymorphisms are revealed (Figure 4). In Figure 4A the DNA of the Carlsberg lager strain BK 2246 is compared to the Tuborg lager strain BK 2224 after cutting with the restriction endonucleases EcoRI, Sall and EcoRV. All three enzymes yield distinguishable patterns for the two strains and these are designated I and II, respectively. Pattern I of the Carlsberg lager strain lacks a *TyI* carrying 5.5 kb EcoRI fragment present in the DNA of the Tuborg strain. The Carlsberg strain, on the other hand, contains an additional EcoRV (~ 8 kb) and Sall fragment (~ 10 kb) which are not present or very weakly hybridising in the Tuborg strain. Ten of the Bavarian bottom fermenting strains have EcoRI fragment patterns (IIa) which are similar to that of the Tuborg strain (Figure 4B, Table I), while one strain (C83-FP35) has the Carlsberg pattern i.e. lacking the 5.5 kb fragment. In the two strains C83-FP34 and C83-FP37 a slightly longer 6 kb EcoRI restriction fragment hybridises instead of the 5.5 kb band. I designate this as pattern IIb (Figure 4B). Even though the pattern IIa in the Bavarian strains resembles that of the Tuborg strain, one difference has been noticed, namely that the Tuborg strain lacks a (~ 10 kb) Sall band which is present in both the Carlsberg strain and the Bavarian strains (cf. Figure 4A versus B).

When DNA from the top fermenting strains is probed with *TyI*, more complex differences in restriction endonuclease fragment patterns are found. Five EcoRI patterns have been identified among the 9 strains (Figure 4C), namely patterns C83-FP48, -FP49, -FP50, -FP52 and -FP55. While all strains have the 2.7 kb EcoRI fragment in common, *TyI* hybridises to a minimum of 10 bands that vary from strain to strain in fragment size and intensity.

4. DISCUSSION

With two exceptions, all bottom and top fermenting strains used in brewing contain form II of the *RDNI* gene encoding the cytosolic ribosomal RNA species. With the present analysis and that reported previously (20) this *RDNI* allele has been identified in 43 lager strains and 19 top fermenting strains. One ex-

ception is the presence of form I of the *RDNI* gene in a British ale strain (Table II in ref. 20). Form III of this gene, which is characteristic for *S. uvarum*, *S. bayanus* and *S. pastorianus* has been found in a Chinese Brewing strain (Table III in ref. 20). The lager yeasts are equally uniform when the restriction patterns for fragments hybridising with probes for the *HIS4* and *LEU2* genes are analysed. For 43 lager strains it has now been documented that they contain the *HIS4* patterns I and II, known to originate from allelic genes located on two homeologous chromosomes (18). As mentioned above, two strains with hemi- or homozygosity of the chromosome III carrying pattern II are known to be lager yeasts of production quality (20, 31, 32). The *LEU2* gene is located on the same chromosome arm as *HIS4*. This chromosome region reveals the same Sall restriction fragment pattern in all investigated lager yeasts (fragments III + IV), when probed with the 3' part of the *LEU2* gene from a genetic standard strain, irrespective of whether the two alleles of the *HIS4* region are present in the heterozygous state or not. Of the nine top fermenting strains analysed in this paper the *HIS4* region on chromosome III had the same pattern I, which is characteristic for the genetic standard strains. This pattern was associated in seven strains with two Sall fragments hybridising to the *LEU2* probe (fragments II + III), while two strains showed patterns in which fragment III was combined with two restriction fragments of lower molecular weight (7 and 5 kb). Fragments II + III may be identical to the two Sall fragments detected by KEIDING (15) in strain A using the same probe. The fact that HOLMBERG (12) did not detect hybridisation of this probe to DNA of lager yeast can be attributed to a higher stringency of hybridisation.

Genetic uniformity of the lager brewing strains is understandable, if they all originated from one of the clones isolated by EMIL CHR. HANSEN in 1883 (31). On the other hand some genetic changes are expected to have taken place over the last 100 years as a result of selections carried out during propagation by different breweries and brewing research laboratories. Different brewing strains have different fermentation properties which provide different taste and satisfy different technological requirements. Us-

ing the *Ty1* transposing element as a probe significant genetic variation between the Carlsberg and Tuborg lager strains was uncovered, two strains known to produce beer with significantly different ester content.

Also among the lager strains in the collection of the Wissenschaftliche Station für Brauerei in Munich the *Ty1* probe could distinguish three different genotypes. Among the top fermenting strains analysed in this work no less than five genotypes with different *Ty1* element distributions were found. Further work is necessary to establish the distribution and chromosomal location of the *Ty1* and other *Ty* elements in the bottom and top fermenting strains. Since the restriction endonuclease fragment patterns of *Ty* elements hybridising to *Ty1* are that similar in bottom fermenting strains it rises the question of the frequency of transposition of these elements in brewing strains and the effects of transposition on strain stability. The high number of genotypes recognized by the *Ty1* probe among the top fermenting yeasts could be due to a more frequent transposition or a greater number of original genotypes which contributed to the collection of ale strains. In view of the variations in brewing strains that can be revealed by the *Ty* probe, this probe has an obvious potential for the identification of existing strains. In addition, it is expected to be useful in the characterization and breeding (31, 32) of improved brewing strains.

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