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ORIGINAL PAPER

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Isolation and properties of genetically defined strains of the methylotrophic yeast *Hansenula polymorpha* CBS4732

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Abstract Genetically defined strains of the yeast *Hansenula polymorpha* were constructed from a clone of *H. polymorpha* CBS4732 with very low mating and sporulation abilities. Mating, spore viability, and the percentage of four-spore-containing asci were increased to a level at which tetrad analysis was possible. Auxotrophic mutations in 30 genes were isolated and used to construct strains with multiple markers for mapping studies, transformation with plasmid DNA, and mutant screening. Various other types of mutants were isolated and characterized, among them mutants that displayed an altered morphology, methanol-utilization deficient mutants and strains impaired in the biosynthesis of alcohol oxidase and catalase. Also, the mutability of *H. polymorpha* CBS4732 vs *H. polymorpha* NCYC495 was compared. The data revealed clear differences in frequencies of appearance and mutational spectra of some mutants isolated. Many of the mutants isolated had good mating abilities, and diploids resulting from their crossing displayed high sporulation frequencies and high spore viability. Most of the markers used revealed normal Mendelian segregation during meiosis. The frequency of tetratype spore formation was lower than in *Saccharomyces cerevisiae* suggesting a lower frequency of recombination during the second meiotic division. The properties of genetically defined strains of *H. polymorpha* CBS4732 as well as their advantages for genetics and molecular studies are discussed.

Keywords *Hansenula polymorpha* · Methylotrophic yeast · Genetic analysis · Mutant isolation

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Introduction

The methylotrophic yeast *Hansenula polymorpha* is an attractive model organism for various fundamental studies, e.g. on the genetic control of enzymes involved in methanol metabolism (de Koning et al. 1987), peroxisome function and biogenesis (van der Klei et al. 1995), nitrate assimilation (Avila et al. 1998), and resistance to heavy metals and oxidative stress (Mannazzu et al. 2000). A characteristic feature of *H. polymorpha* is its thermotolerance: cells can grow at temperatures up to 49 °C, and may even be able to survive pasteurization (Teunison et al. 1960). Furthermore, *H. polymorpha* is widely applied as host for production of foreign proteins (Gellissen and Melber 1996; van Dijk et al. 2000). Nevertheless, the genetic background of the strains used and knowledge of the control of basic cellular processes such as cell division, mating, and sporulation are still largely unclear.

Also, the taxonomic status of *H. polymorpha* has been the subject of debate. Proposed reclassification of the genus *Hansenula* involved the transfer of this organism to the genus *Pichia* and the name *Pichia angusta* was adopted (Kurtzman 1984). This new nomenclature is not quite current and rarely used in scientific reports. It is proposed to indicate various strains of methylotrophic yeast newly isolated from *Opuntia* cacti as *Pichia angusta* and to use *H. polymorpha* for one sibling group of genetically employed strains (Naumov et al. 1997).

So far, most genetic work has been done with three *H. polymorpha* strains, DL-1, CBS4732, and NCYC494. These strains have independent origins, different features and unclear relationships. The strain DL-1 (synonymous with ATCC26012, NRRL-Y-7560) was isolated from soil

(Levine and Cooney 1973). There are no data available on the ability of DL-1 strains to mate and sporulate. According to DNA/DNA hybridization and karyotyping, strain DL-1 is very similar to strain CBS4732 (Naumov et al. 1992). Strain NCYC495 (synonymous with CBS1976, ATCC 14754, NRRL-Y-1789, VKM-Y-1397) was first isolated from spoiled orange juice and described as *Hansenula angusta* (Wickerham 1951). This strain is homothallic haploid, has good mating and sporulation abilities and has been used for the isolation of auxotrophic and temperature-sensitive mutants affected in methanol metabolism (Gleeson et al. 1984; Gleeson and Sudbery 1988). Strain CBS4732 (synonymous with ATCC34438, NRRL-Y-5445, CCY38–22–2) was isolated from soil in Brazil (de Morais and Maia 1959). These cells grow well on methanol-containing media; moreover, they also perform well in continuous cultures (van Dijken et al. 1976) and in large-scale fermentation (Gellissen and Melber 1996). For these reasons, strain CBS4732 has been widely used in biochemical and physiological studies. Nevertheless, serious problems were encountered with its semisterility and inability to sporulate (Gleeson et al. 1984).

Two additional genetic lines of *H. polymorpha* with a hybrid origin have been reported. The strains described by Bodunova et al. (1986) have as parentals two auxotrophic non-mating mutants that originated from strains NCYC494 and ML-3, probably derived from strain DL-1. These strains have been crossed by protoplast fusion. From the resulting diploid, several mitotic segregants with copulation ability were isolated upon UV irradiation and used for intertetrad breeding. These resulting strains possessed good mating ability and a high percentage of sporulation. Strains used in the Veenhuis group (Groningen, The Netherlands) are designated as NCYC495 but in fact originate from back-crossings of mutants isolated from the CBS4732 wild-type (WT) strain (Cregg et al. 1990), with NCYC495 auxotrophs (Waterham et al. 1992). In the last few years these strains have become popular because they have several advantages: (1) high frequencies of mating and sporulation, (2) good growth on methanol-containing media, (3) thermostability, (4) well- developed systems for electrotransformation (Faber et al. 1994). Several unusual properties have been reported for strains having a hybrid origin, e.g. serious deviations from the normal Mendelian segregation of genetic markers during meiosis (Bodunova et al. 1986) and mating and sporulation at 43 °C (Waterham et al. 1992). This emphasizes the need to use strains in genetic studies that are highly isogenic according to their progeny. Here we report the construction of genetically defined strains of *H. polymorpha* CBS4732. They contain various auxotrophic mutations, and show good mating ability, normal meiosis, high percentage of sporulation and good spore viability. In addition, we describe their use in mutant isolation and analysis. The availability of a *H. polymorpha* CBS4732 breeding stock allows greater flexibility in genetic manipulation, rendering it a more amenable genetic system for basic research.

Materials and methods

Strains, media, and growth conditions

All strains described had as an original parental a clone of *H. polymorpha* CBS4732, kindly supplied by Dr. L. Silhankova (Prague). This strain has unknown ploidy and very low mating and sporulation rates. By means of successful inbreeding and selection procedures, described in the Result section, strain CBS4732s with improved genetic abilities was obtained. This strain was subjected to mutagenic treatment, and some of the isolated auxotrophic mutants were utilized for the construction of highly isogenic haploid strains useful for mutagenic treatment and genetic analysis. The strains used are CBS4732s, A3 *ura3–20*, 5C-HP1*56 ade2–88*, 22-HP41 *ade1–37 arg 1–11*, 14B-HP156 *leu2–2 cat1–14*, and 1D-HP8 *ura2–1 leu2–2 met4–22*.

Strains were cultured in a rich YPD medium (1% yeast extract, 2% peptone, 2% glucose) or a minimal (MIN) medium [0.67% yeast nitrogen base without amino acids (YNB), 2% glucose]. Auxotrophic requirements were determined by using diagnostic or dropout (DO) media (0.67% YNB, 2% glucose, supplemented with selected combinations of the required amino acids and nucleic-acid bases added to 50 μg/ml). Growth of strains on media with different carbon sources was investigated by using several synthetic complete (SC, 0.67% YNB, supplemented with all required amino acids and nucleic-acid bases) media: SCG (SC with 2% glucose), SCM (SC with 1% methanol), SCE (SC with 1% ethanol) or SCGCR (SC with 1% glycerol). The sporulation media SPOMAL and SPOME used in experiments for induction of mating and sporulation contained 2% maltose (SPOMAL) and 5% malt extract (SPOME), respectively. All solid media were made with 2% agar. Yeast cells were cultivated at 37 °C. In experiments for induction of mating and sporulation, the strains were cultivated on SPOMAL or SPOME media at room temperature.

Analysis of genetic markers

Auxotrophic markers were scored by replica-plating to appropriate DO media which were screened after 1–2 days at 37 °C. Ade$^-$, Leu$^-$, Ura$^-$, Met$^-$, Arg$^-$ and Lys$^-$ phenotypes indicates inability to grow on DO media lacking adenine, leucine, uracil, methionine, arginine and lysine, respectively. The phenotype Mut$^-$ was scored by replica-plating of strains onto SCM plates. After 2 days of cultivation, strains unable to grow were designated as Mut$^-$. The phenotypes Eth$^-$ (ethanol-utilization deficient) and Gcr$^-$ (glycerol non-utilization) were assayed by the same approach. The phenotype Cat$^-$ (absence of catalase activity) was determined in colonies using a 10-ml overlay of 1% H_2O_2 per plate. Colonies that failed to form O_2 bubbles due to the absence of catalase activity were designated as Cat$^-$. The phenotype Aox$^-$ (absence of alcohol oxidase activity) was scored using the plate assay described previously (Titorenko et al. 1995). Methods for discrimination of morphological mutants Rgh and Rap are described in the text.

Induction and isolation of mutants

N-methyl-*N*′-nitro nitrosoguanidine (NTG) mutagenesis was performed as described (Cregg et al. 1990). For the isolation of auxotrophic mutants, the nystatin enrichment procedure was used following NTG mutagenesis (Sanchez and Demain 1977). For UV-induced mutagenesis, cells were irradiated directly on YPD media with UV doses allowing survival of 1–5%. YPD plates with NTG- and UV-treated cells were incubated for 3–6 days and the resulting colonies were replica-plated onto corresponding MIN or SC media (for the isolation of auxotrophic mutants) or SCM medium (for the isolation of *Mut*$^-$ mutants).

Other methods and procedures

Multiple crosses were carried out using plate-to-plate mass hybridization. The strains with complementary nutritional markers were plated as parallel lines onto two YPD plates, grown overnight, crossed by velvet replica-plating and transferred onto SPOMAL or SPOME plates. These plates were cultivated at room temperature for 2 days and replica- plated by filter paper onto selective MIN media. After 2 days of cultivation at 37 °C, only the resultant hybrid strains showed growth.

Diploid cells resulting from the mating crosses were purified by streaking for single colonies, tested for maintenance of the corresponding phenotype, and plated onto SPOMAL or SPOME media. After 3–4 days of cultivation at room temperature, a pale pink color appeared concurrent with the formation of four-spored hat-shaped asci. Random spore analysis by diethyl ether killing was performed by suspending sporulating diploid cells in 1 ml distilled water in a 2-ml Eppendorf tube and adding 1 ml of diethyl ether (Dawes and Hardie 1974). Tubes were shaken for 15–20 min, diethyl ether removed and subsequently various cell dilutions were plated onto YPD plates. After 3–4 days cultivation at 37 °C, visible colonies were picked and analyzed for genetic markers. Tetrad dissection, determination of tetrad types, and calculation of map distances was performed as recommended by Zakharov et al. (1984). *D*(cM), the distance in centimorgans, was calculated according to the formula $D=6NPD+T/2(PD+NPD+T)\times100$, where PD is the number of tetrads with parental ditype, NPD the tetrads with non-parental ditype, and T the number of tetratype tetrads. Two genes were considered linked in case of PD>NPD and T<2/3.

Table 1 Phenotypes and number of defined complementation groups (genes) among auxotrophic mutants isolated by NTG mutagenesis of the wild-type strains of *Hansenula polymorpha* CBS4732 and *H. polymorpha* NCYC495 (according to Gleeson and Sudbery 1988) and *Hansenula polymorpha* CBS4732

Mutant phenotype	NCYC 495		CBS 4732	
	Number of isolates	Defined genes	Number of isolates	Defined genes
Ade–	106	12	115	11
Met–	52	10	47	6
Arg–	12	5	12	4
Asp–	11	3	0	0
His–	6	6	0	0
Ura–	5	3	38	3
Leu–	1	1	4	2

Results

Isolation and analysis of auxotrophic mutants

After prolonged cultivation on SPOMAL medium, the parental *H. polymorpha* CBS4732 strain produced a small number (4–6%) of four-spored asci. After their dissection by micromanipulation the spore viability was approximately 2%. Full tetrads were not observed. The meiotic segregants were plated on SPOMAL medium and one of them, designated as CBS4732s, displayed an increased sporulation level concurrent with ~50% spore viability. The mitotically grown cells of CBS4732s were used for the isolation of auxotrophic mutants by NTG mutagenesis followed by nistatin enrichment. In three independent experiments, auxotrophic mutants appeared with a frequency of 1.8%. In total, a collection of 216 auxotrophs was obtained; their spectra are presented in Table 1. All mutants had recessive phenotypes. Mutants belonging to the same phenotypic class were crossed in all possible combinations and allocated to corresponding complementation groups (Table 2). The auxotrophic phenotypes and the number of complementation groups obtained in this work and those reported for the NCYC495 genetic background (Gleeson and Sudbery 1988) are presented in Table 1. In both experiments NTG was used as mutagen at similar survival rates, thus allowing comparison of the spectra of mutations. Adenine-requiring mutants occurred most frequently in both strains, but the absence of mutants requiring aspartate and histidine was characteristic for the CBS4732s strain. Mutants auxotrophic for aspartate, histidine, proline and cysteine were isolated additionally in strains A3, 1D-HP8 and 22-HP41 by irradiation with UV light and allocated to corresponding complementation groups (data not shown). As a result the number of identified auxotrophic genes amounted to 30.

For some of the genes it was possible to establish the nature of the encoded proteins. Mutants affected in the *ADE*1 and *ADE*2 genes developed a red-purple color after 5–7 days of incubation on YPD plates. *ADE*2 mutants appeared twice as frequently as *ADE*1 mutants (Table 2). Furthermore, for *ADE*2 alleles interallelic complementation was observed. Enzyme assays in crude extracts revealed that in the *ade1–37* mutant phosphoribosyl-

Table 2 Number of genes and alleles defined by complementation tests and recombination analysis of auxotrophic mutants isolated from strain CBS4732s

Gene	Alleles	Gene	Alleles	Gene	Alleles	Gene	Alleles	Gene	Alleles
*ADE*1	6	*MET*1	5	*ARG*1	2	*URA*1	3	*LEU*1	1
*ADE*2	10	*MET*2	10	*ARG*2	3	*URA*2	3	*LEU*2	3
*ADE*3	17	*MET*3	11	*ARG*3	1	*URA*3	18		
*ADE*4	9	*MET*4	3	*ARG*4	1				
*ADE*5	12	*MET5*	2						
*ADE*6	3	*MET6*	1						
*ADE*7	1								
*ADE*8	4								
*ADE*9	2								
*ADE*10	1								
*ADE*11	1								

aminoimidazole-succinocarboxamide synthetase activity was absent and that in the *ade2–88* mutant no carboxy aminoimidazole ribonucleotide (CAIR):aspartate ligase activity could be found. In the *ade1–37 ade2–88* double mutant both enzyme activities were undetectable (V. Domkin, St. Petersburg University, Russia, personal communication). These data suggest that the *ADE*1 and *ADE*2 genes of *H. polymorpha* CBS4732 are orthologues of the *ADE*1 and *ADE*2 genes of *Saccaromyces cerevisiae*, respectively. In addition, it was observed that the *S. cerevisiae URA3 and LEU2* genes complemented the *ura3–20* and *leu2–2* mutations, respectively (data not shown), suggesting that they code for the enzymes orotidine-monophosphate decarboxylase and β-isopropyl malate dehydrogenase, respectively.

Mutants with altered morphology

The majority of colonies that arose after mutagenic treatment displayed a characteristic hemispherical shape with a smooth circular outline (not shown). These colonies consisted of oval, sometimes spherical yeast cells (Fig.1A, inset). We designated these two wild-type phenotypes (smooth colonies/ oval cells) as Rgh$^+$. After UV mutagenesis of strain 14B-HP156, 27 mutant colonies with irregular outline and rough surface were obtained out of approximately 23,500 surviving colonies (not shown). Cells of these mutants sediment rapidly in water; light microscopic analysis revealed that most of them were organized in chains of undivided cells (Fig. 1A). The majority of these chains consisted of four cells, but more elongated chains were also observed. Most of the chains were linear, but cell aggregates with several lateral chains were observed too. This mutant phenotype (rough colonies, chains of cells) was designated as Rgh$^-$ (from *rough*). In tetrad analysis a strict monogenic segregation, 2 Rgh$^+$: 2 Rgh$^-$, was established for the mutants analyzed. In all cases the phenotypes smooth colonies/oval cells (Rgh$^+$) and rough colonies/chains of cells(Rgh$^-$) cosegregated, suggesting that they were determined by single nuclear mutation(s). Complementation and recombination analysis allocated all morphological mutants into four genes: *RGH1* (3 alleles), *RGH2* (5 alleles), *RGH3* (15 alleles) and *RGH4* (2 alleles). The gene *RGH3* appeared to be linked to the *LEU2* locus (35.7 cM).

A second type of rough-colony -forming mutant, containing elongated, pseudohyphal cells, was isolated and designated as *rpm1* (from *r*ough, *p*seudo-*m*ycelial) (Fig. 1B). Remarkably, cells of *rpm1* penetrated into the agar surface of YPD plates by invasive growth (not shown).

A third type of morphological mutant formed colonies of very small size. Such colonies (0.5–1 mm in size even after 8–10 days of cultivation) appeared frequently after mutagenic treatment. In most of the cases, the normal colony size was restored after several successive cycles of plating on rich media. Two mutants, designated as *rap1* and *rap2* (from *rap*id growth), stably maintained their small colony size. After repeated plating onto YPD medium the colonies of these strains only slightly increased in size but never exceeded a diameter of 2 mm. The wild-type "large-" and mutant "small-" sized colonies could be easily distinguished by eye after 24 h of growth on YPD plates. The *rap1* mutation appeared to be tightly linked to the *URA3* locus (1.0 cM). Furthermore, *rap2* was linked to the *LYS1* gene (7.4 cM) The strains carrying *rap1* and *rap2* mutations were very tedious for genetic analysis because in the homozygous state they showed strongly decreased mating and sporulation phenotypes.

Methanol-utilization-deficient (Mut$^-$) mutants

In order to identify genes essential for growth on methanol, various Mut$^-$ mutants were isolated at very high frequencies after UV irradiation of strain 5C-HP156. A compari-

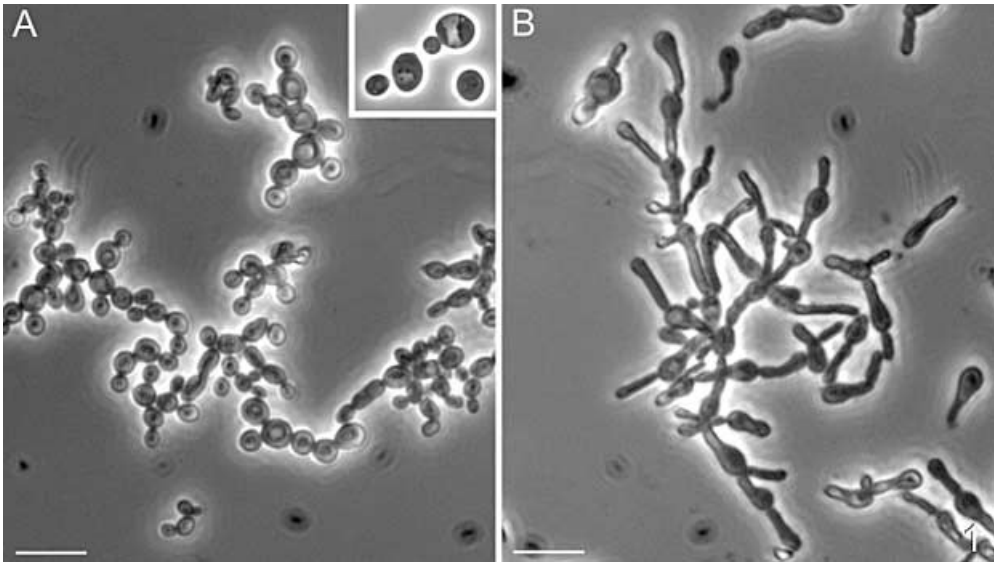

Fig. 1A, B Morphology of mutant *H. polymorpha* cells. Phase-contrast images to demonstrate the morphology of glucose-grown *rgh1* cells (**A** chains of cells) and *rpm1* cells (**B** pseudohyphal cells). The morphology of wild-type cells is shown as control (**A** *inset*). *Bar* 10 μm

Table 3 Frequency of mutants, defective in methanol-utilization (Mut^-) and mutants, deficient in alcohol oxidase (AO^-) and catalase (CAT^-) activities in strains belonging to *H. polymorpha* CBS4732 (strain 5C-HP156), this study, and *H. polymorpha* NCYC495 strain L-1) (Vallini et al. 2000). *ND* Not determined

	5C-HP156	L-1
Mutant phenotype		
Mut^-	262	65
Mut^- Eth^-	105	2
Mut^- Gcr^-	87	2
Mut^- Eth^- Gcr^-	114	1
AO^-	25	ND
CAT^-	20	ND
Mutants allelic to *aox1* gene	12	1
Mutants allelic to *cat1* gene	20	2
Number of colonies tested	6,525	30,000

son of our results with those obtained for the NCYC495 strain L-1 (Vallini et al. 2000) demonstrated the advantage of using CBS4732 strains for the isolation of Mut^- mutants (Table 3). In both cases UV irradiation at 1–5% survival was used as mutagen. Mut^- strains, also unable to grow on ethanol (Mut^- Eth^-), glycerol (Mut^- Gcr^-) or both (Mut^- Eth^- Gcr^-), were also identified but were not further studied. It is now generally accepted that Mut^- mutants appear as a result of mutations in at least two different levels, namely: (1) genes encoding enzymes involved in methanol metabolism (de Koning et al. 1987), and (2) genes essential for peroxisome biogenesis and function (*pex* genes; van der Klei et al. 1995). Both groups of mutant were identified among the strains belonging to our Mut^- collection. All Mut^- mutants were crossed to the wild-type strains and all but two of them had a recessive phenotype. The observed two exceptions revealed clear dominance of their Mut^- phenotype. Transmission electron microscopy of ultrathin sections of diploid cells, heterozygous for the mutations studied, did not reveal the presence of peroxisomes in methanol-grown cells of the dominant mutants (not shown), indicating that they are true *pex* mutants.

Mutants impaired in alcohol oxidase and catalase activities

The stable Mut^- clones were further analyzed for alcohol oxidase and catalase activities. Peroxisome-bored and catalase proteins are key enzymes involved in methanol metabolism of methylotrophic yeasts (van der Klei et al. 1995). The structural genes for alcohol oxidase (*AOX1)* and catalase (*CAT1)* have been cloned and sequenced (Ledeboer et al. 1985; Didion and Roggenkamp 1992). The availability of strains carrying disrupted *AOX1* or *CAT1* genes provided the possibility to identify mutants in these genes by complementation tests and to obtain additional genes involved in biosynthesis (e.g. cofactor binding, octamerization).

Catalase- and alcohol-oxidase- activity-deficient mutants appeared at similar frequencies (Table 3). Complementation tests demonstrated that all CAT^- mutants were affected in the structural *CAT1* gene. In tetrad analysis for 252 out of 263 tetrads a strict monogenic segregation (2^+:2^-) was found. This analysis also revealed that *CAT*1 is linked to the *URA3* locus (28.8 cM).

About half of the isolated alcohol-oxidase-deficient mutants were unable to mate. These were excluded from our analysis. The other alcohol oxidase mutants had very low mating abilities, and their mating was improved after several rounds of back-crossing with the wild-type strain. Complementation tests revealed that all alcohol oxidase mutant strains were affected in the structural *AOX1* gene (Table 3). In tetrad analysis of diploids heterozygous for the *aox1* mutation, normal 2^+: 2^- segregation was obtained for 196 tetrads analyzed. The *AOX1* gene appeared to be linked with the *ADE*2 locus (39.5 cM). In further experiments, 64 additional alcohol oxidase mutants were isolated after UV irradiation of strain 14C-HP156, and complementation tests revealed two additional genes involved in alcohol oxidase biosynthesis, designated *AOX2* and *AOX3*. Mutations *aox2–1* and *aox3–1* displayed strict monogenic segregation. On Western blots of crude extracts prepared from *aox1–1, aox2–1* and *aox3–1* mutant cells, using α-alcohol oxidase antibodies, alcohol oxidase protein was not detectable. The peroxisome number and morphology were studied by electron microscopy. In *aox1–1* cells the number of peroxisomes was unchanged but the organelles were smaller in size (not shown). The number of peroxisomes had increased three- to four-fold in the *aox2* mutant (Fig. 2B); however, these were of very small size. In *aox3* cells, up to three small peroxisomes were observed per cell (Fig. 2C).

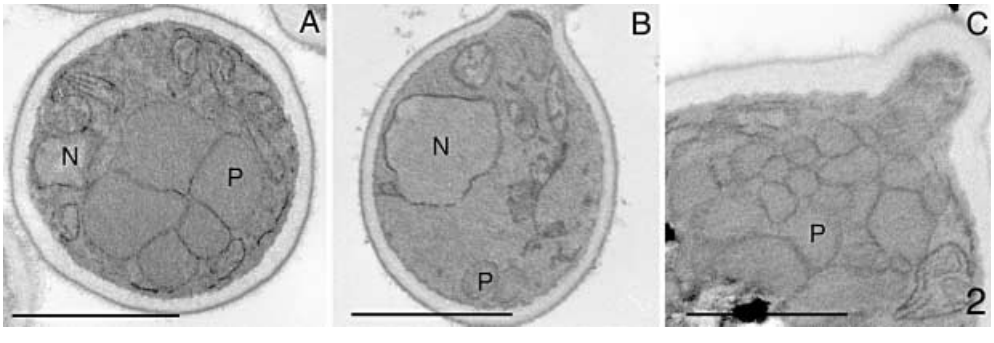

Fig. 2 Electron micrographs prepared from $KMnO_4$-fixed cells of the wild-type strain 5C-HP156 (**A**) and mutants *aox2* (**B**), *aox3* (**C**). *N* Nucleus, *P* peroxisome. *Bar* 0.5 μm

Table 4 Meiotic segregation of *Hansenula polymorpha* CBS4732 alleles

Allele pair	2+:2–	3+:1–	3–:1+	4+:0–	4–:0+	Irregular tetrads (%)
ade1–37:ADE1	28	1	2	0	1	12.5
ade2–88:ADE2	225	12	4	3	1	8.2
ade3–5:ADE3	14	1	0	0	0	6.7
met1–11:MET1	32	0	5	1	2	20.0
met3–2:MET3	58	2	0	1	1	6.5
met5–29:MET5	25	3	1	0	0	13.8
Leu2–2:LEU2	248	12	6	1	2	7.8
arg1–11:ARG1	15	0	1	0	0	6.3
ura3–20:URA3	115	9	5	1	2	12.9
ura2–1:URA2	49	1	3	1	0	9.3
cat1–14:CAT1	252	7	5	1	3	5.9
aox1–1:AOX1	196	3	2	3	2	4.8
aox2–1:AOX2	25	1	1	0	0	7.4
aox3–1:AOX3	17	1	0	0	0	5.5

Table 5 Tetrad analysis. Data (in numbers) were scored from tetrads segregating 2+:2– for the markers. *Linked genes

Gene pair	Parental ditype	Nonparental ditype	Tetratype tetrads	Frequencies of tertratype tetrads	Distance in centimorgans
ade1-arg1	25	1	23	0.469	29.6*
ade1-ura3	15	8	53	0.732	66.4
arg1-ura3	17	2	20	0.512	41.0
ade2-ura3	30	32	103	0.624	89.4
ade2-leu2	33	28	109	0.641	81.4
leu2-ura3	30	33	75	0.543	98.9
ade2-cat1	32	36	105	0.606	92.7
leu2-cat1	28	39	98	0.593	100.6
ade1-cat1	5	4	35	0.795	67.0
arg1-cat1	8	5	32	0.711	68.8
ura3-cat1	124	7	79	0.346	28.8*
ura3-rap1	98	0	2	0.34	1.0*
cat1-rap1	54	3	43	0.43	30.4*
arg1-rap1	15	1	23	0.589	37.1*
aox1-ade1	9	11	26	0.565	100.0
aox1-ade2	93	11	78	0.428	39.5*
aox1-arg1	12	7	27	0.586	75.0
aox1-leu2	31	21	92	0.638	75.7
aox1-ura3	42	56	98	0.5	111.2
aox1-cat1	35	40	107	0.587	161
rgh1-ade2	10	7	26	0.604	79.0
rgh1-leu2	21	11	37	0.536	74.6
rgh2-ade2	7	5	16	0.571	82.0
rgh3-leu2	15	1	19	0.542	35.7*

Meiotic segregation

In sporulating diploid cultures a normal Mendelian segregation was observed for most of the markers in random spore or tetrad analysis. The frequencies of irregular tetrads varied for the mutations studied, but only in a few pairs exceeded 10% (Table 4). Similar deviations from normal segregation rates have been observed for some markers in random spore analysis. The reasons for the observed deviations are unclear; possibly, some cases are due to aneuploidy events. For some pairs of markers a genetic linkage was found, but the number of linked genes was not high (Table 5). Remarkably, no centromere-linked markers were discovered. The average frequency of tetratype formation was estimated at 0.56, and this is lower than the normal tetratype frequency of 0.67 reported for *Saccharomyces cerevisiae*. This is probably due to the lower frequency of recombination during the second meiotic division.

Discussion

Recently, the yeast *H. polymorpha* has become the subject of increasing interest for fundamental studies and industrial applications. However, the strains used nowadays

greatly vary in their characteristics with respect to origin and taxonomy. Moreover, a common feature of all strains used so far is the small number of available auxotrophic markers: mainly *ura3* and *leu2* (designated as *leu1* in NCYC495 strains). This limitation has obviously hampered strain construction and improvement by classical genetics. Only one strain has been reported (A16), the growth properties of which were improved by the use of conventional genetic techniques (Veale et al. 1992).

Here, we report our initial studies on the development of genetically defined strains of the yeast *H. polymorpha* CBS4732 and summarize some data about their properties. It has been reported that CBS4732 strains are semisterile and unsuitable for genetic analysis (Gleeson et al. 1984). This was probably due to the nature of the strain used. Our improvements are related to the availability of a CBS4732 strain that was able to sporulate (albeit at low rates) and the selection of a meiotic segregant (CBS4732s) with improved sporulation rates.

Several types of mutants (auxotrophic, morphological, impaired in methanol metabolism and/or peroxisome functions) have now been isolated by NTG- and UV-induced mutagenesis. These mutant collections are valuable for the further study of various *H. polymorpha* functions. The auxotrophic mutants have been used to establish complementation tests, determine meiotic segregation ratios, and for the creation of strains with multiple auxotrophic markers. These strains are ideal for genetic analyses as they show good mating properties, sporulate well and have high spore viability. Furthermore, the segregation of genetic markers is normal. Each of them carry *leu2* and *ura3* mutations as selective markers for DNA transformation experiments in addition to another auxotrophic marker(s). The meiotic segregants obtained in the course of this work varied in their ability to grow on methanol media at physiological (37 °C) as well as at elevated temperatures (44 °C). By selection and further utilization of strains with desired phenotypes it was possible to improve their growth characteristics and to establish new strains.

The isolated morphological mutants together with the red-pigmented *ade1* and *ade2* strains are very suitable for rapid isolation of recombinant meiotic segregants by visual selection. The mutants belonging to the *rgh1–rgh4* group (forming chains of cells) probably had a defect in cell separation and could be useful for studying the mechanisms of septum formation and cell separation. No data on the ability of *H. polymorpha* to form pseudo-mycelia have been reported thus far (Kreger-van Rij 1984). The *rpm1* mutant is the first mutant capable of constitutive filamentous and invasive growth. The properties of this mutant suggest that *H. polymorpha* cells can undergo filamentous and invasive growth differentiation, which opens novel possibilities for investigations of these processes.

The data obtained in the course of our mutant isolation procedures revealed some features of mutational processes in *H. polymorpha*. Generally, the frequency of mutant appearance in *H. polymorpha* is lower than reported for *S. cerevisiae* (Gleeson et al. 1984; Gleeson and Sudbery 1988). This is probably due to the fact that in these experiments mainly large-sized colonies which arose after mutagenic treatment were selected and studied. We observed that the most of the induced mutants were among the very small-sized (Rap^-) colonies. The work with such colonies was tedious and slow because they needed additional rounds of replating and back-crosses. We compared our results with those describing mutation frequencies of *H. polymorpha* NCYC495 (Gleeson and Sudbery 1988; Vallini et al. 2000). This approach is feasible because the same mutagens were used at similar survival rates in both experiments. The data revealed no significant differences between the two strains regarding the frequencies of appearance of many types of mutants. The low frequencies obtained for L-1 (Vallini et al. 2000) suggests that this strain is autodiploid. Nevertheless clear examples of mutational specificity were observed for the CBS4732 and NCYC495 strains. These strains differ in their ability to produce certain types of mutants and the distribution of alleles among the genes. Such a bias has been observed for other methylotrophic yeasts, e.g. *Pichia methanolica* (*Pichia pinus*) MH4 (Tolstorukov et al. 1977) and *Candida boidinii* (Lahtchev et al. 1992).

Remarkable results were obtained for mutants lacking alcohol oxidase activities. In our experiments with CBS4732, these mutants were mainly affected in the *AOX1* structural gene. The two deviant mutants (*aox2* and *aox3*) appeared with very low frequencies. Recently, a large collection of alcohol-oxidase-deficient mutants has been isolated after NTG mutagenesis of a strain belonging to the Veenhuis group (van Dijk et al. 2001). Complementation tests revealed that over 50% of these were allelic to the *AOX1* gene. Within these Aox^- strains, several mutants impaired in alcohol oxidase assembly were obtained. These results indicate that for the isolation of larger mutant spectra in *H. polymorpha* it is preferable to use several strains with independent origins as well as different types of mutagens.

The strains described here were suitable for genetic analysis because of their high mating and sporulation abilities. Diploids were easily obtained and complementation tests were effective, allow the testing of up to 64 combinations per plate. The ability of *H. polymorpha* CBS4732 cells to mate in all possible combinations facilitated these experiments.

The spore viability of diploids was high and facilitates efficient tetrad and random spore analysis. Tetrad analysis is possible for *H. polymorpha* CBS4732 and has been used successfully to study the mechanisms of plasmid integration (Bogdanova et al. 1995). Some differences between sporulating cultures, originating from the CBS4732 and NCYC495 genetic background, probably exist. The ascus membrane of NCYC495 was shown to be resistant to enzymatic digestion. Furthermore, the strong adhesion between ascospores made their separation very difficult (Gleeson and Sudbery 1988). We have not had these problems with CBS4732 ascospores, which could be easily dissected without prior enzymatic treatment (Bogdanova et al. 1995). Nevertheless the small size of the spores made tetrad analysis slow and tedious so that only 24 gene

pairs were studied. Random spore analysis was used for the investigation of the other 56 gene pairs. The number of linked genes was considerably low and, because of the absence of centromere-linked markers, hampered the construction of a *H. polymorpha* genetic map.

The observed regular meiotic segregation of genetic markers is obviously very important for further work with the *H. polymorha* CBS4732 genetic stock. Nevertheless, the obtained deviations from normal monogenic segregation suggests that some of the chromosomes in the strains studied were probably duplicated. The data from pulse gel electrophoretic separation of *H. polymorpha* chromosomes also showed that some of the chromosome bands were doubled (Naumov et al. 1992). This indicated that *H. polymorpha* is probably tolerant to aneuploidy events. Aneuploidy is one possible explanation for the occasionally observed low mutant frequencies.

It is relevant to note that the strains reported here are highly isogenic according to their progeny. The other isogenic strain, NCYC495, has unclear taxonomic relations to CBS4732. According to an early study, they represent two very close, but genetically distinct strains (Naumov and Shchurov 1987). Recent new data indicate that these strains belong to one systematic group (Naumov et al. 1997). The comparison of our results with those reported by Gleeson and Sudbery (1988) suggests that some degree of heterozygosity of their genomes probably exist. The new genetic breeding stock of CBS4732 strains is obviously very useful for further investigations of *H. polymorpha* functions and allows future comparative genetic and systematic investigations.

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