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ORIGINAL PAPER

Physiological and metabolic diversity in the yeast *Kluyveromyces marxianus*

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Abstract *Kluyveromyces marxianus* is homothallic hemiascomycete yeast frequently isolated from dairy environments. It possesses phenotypic traits such as enhanced thermotolerance, inulinase production, and rapid growth rate that distinguish it from its closest relative *Kluyveromyces lactis*. Certain of these traits, notably fermentation of lactose and inulin to ethanol, make this yeast attractive for industrial production of ethanol from inexpensive substrates. There is relatively little known, however, about the diversity in this species, at the genetic, metabolic or physiological levels. This study compared phenotypic traits of 13 *K. marxianus* strains sourced from two European Culture Collections. A wide variety of responses to thermo, osmotic, and cell wall stress were observed, with some strains showing multi-stress resistance. These traits generally appeared unlinked indicating that, as with other yeasts, multiple resistance/adaptation pathways are present in *K. marxianus*. The data indicate that it should be possible to identify the molecular basis of traits to facilitate selection or engineering of strains adapted for industrial environments. The loci responsible for mating were also identified by genome sequencing and PCR analysis. It was found that *K. marxianus* can exist as stable haploid or diploid cells, opening up additional prospects for future strain engineering.

Keywords *Kluyveromyces marxianus* · Industrial yeast · Physiology · Stress · Ploidy

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Introduction

Modern yeast biotechnology places a large emphasis on developing new traits in existing yeasts, or in identifying yeast species with novel traits or properties (Porro and Branduardi 2009). Currently, the majority of yeast biotechnology uses the species *Saccharomyces cerevisiae* (or close relatives) for applications as diverse as vaccine production and wine-making. Limitations with *S. cerevisiae*, however, have led to an increased focus on the potential of so-called non-conventional yeasts, such as *Pichia, Zygosaccharomyces*, and *Kluyveromyces. Kluyveromyces* is a genus

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within the hemiascomycetous yeasts that has been restricted to six species following the reclassification into monophyletic genera based on 26S rDNA sequence (Kurtzman 2003; Lachance 2007). Two species within the genus, *Kluyveromyces lactis* and *Kluyveromyces marxianus*, carry the *LAC12-LAC4* gene pair, responsible for the uptake and subsequent cleavage of lactose into glucose and galactose, and are thus lactose positive (Schaffrath and Breunig 2000; Rubio-Texeira 2006). *K. lactis* was selected as a model for genetic analysis of lactose positive yeasts and has been extensively studied at a molecular level (Schaffrath and Breunig 2000; Fukuhara 2006). In contrast, the sister species, *K. marxianus*, is less well studied at the molecular level but has been adopted by industry for applications ranging from biomass production to bioremediation (Fonseca et al. 2008; Lane and Morrissey 2010). Industrial applications of *K. marxianus* take advantage of traits such as rapid growth, thermotolerance, secretion of the enzyme inulinase and production of ethanol. Although these individual traits have been described in many strains of *K. marxianus,* there has been little effort to study the molecular physiology of this yeast, nor to determine the level of phenotypic diversity within the species.

Many of the applications of *K. marxianus* take advantage of specific strains to produce ethanol when growing on substrates containing sugars such as inulin or lactose. The particular interest in lactose as a carbon source arises because it is present at a concentration of 4–5% in whey, traditionally a waste product of the dairy industry. *K. marxianus* is an aerobic yeast and its capacity to generate energy by mixed respiration and fermentation is described as respiro-fermentative metabolism. This dual capacity is common in hemiascomycetous yeasts and appears most highly regulated and sophisticated in *S. cerevisiae* (Piskur et al. 2006). Several regulatory processes contribute to the efficient fermentative capacity of *S. cerevisiae,* most notably the strong "Crabtree effect", which appears to be largely due to carbon catabolite (glucose) repression of genes encoding respiratory enzymes, and the weak "Pasteur effect", which would be inhibition of fermentative metabolism by oxygen (Barnett and Entian 2005). Both *K. lactis* and *K. marxianus* are traditionally classified as "Crabtree negative" yeasts, implying an inability to effectively ferment sugars to ethanol. Nonetheless, the widespread reports of applications of *K. marxianus* in ethanol production suggest that this may not be an entirely accurate classification. A number of recent studies have attempted to understand respiro-fermentative metabolism in hemiascomycetous yeasts (Blank et al. 2005; Merico et al. 2007, 2009; Rozpedowska et al. 2011). Some interesting findings are that the distribution of this trait between species is variable, and at least one reason for the poor fermentative ability of some species may be an inability to maintain redox balance under low oxygen conditions (Merico et al. 2007). Generally, however, these studies used a single representative strain from each species and the extent of physiological variation within a species was not assessed.

Because industrial fermentations typically use complex substrates, other traits that are of general importance are tolerance to high osmolyte concentrations and to cell-wall inhibitors. In *S. cerevisiae*, the stress activated protein kinase Hog1p, is activated by osmotic and other stresses, and this leads to an adaptive response to cope with adverse environmental conditions (Smith et al. 2010; Hohmann 2002; Hohmann et al. 2007). A homologous system has been described in *K. lactis* (Kawasaki et al. 2008) and recently a *HOG1* orthologue was disrupted in *K. marxianus* NBRC 1777, leading to sensitivity to salt and oxidative stress (Qian et al. 2010). Tolerance to cell wall damage in *S. cerevisiae* is mediated by another MAP kinase pathway termed the cell wall integrity (CWI) pathway, and research with *K. lactis* suggests that an orthologous system functions in this yeast (Rodicio et al. 2008; Rodicio and Heinisch 2010). Overall, these findings indicate that similar stress response pathways may operate in *Saccharomyces* and *Kluyveromyces* species, but there are no data on regulatory mechanisms, nor on or genetic and phenotypic variation in these traits in *K. marxianus.* There are also no data to explain the increased thermotolerance of *K. marxianus* over its sister species *K. lactis*.

Early assumptions that conservation of homologous genes implied that identical metabolic and regulatory processes occurred in related yeasts have long been dismissed (Li and Johnson 2010). In addition, however, from work with *K. lactis*, it is clear that significant physiological differences can also occur within the species (Schaffrath and Breunig 2000; Suleau et al. 2005). From the wide spectrum of biotechnological studies of *K. marxianus,* it has

already become apparent that diversity is present within the species. The aim of this current study was to examine phenotypic and physiological diversity in a selection of strains to try to establish the extent of variation within the species.

Materials and methods

Yeast strains

The *K. marxianus* strains used in this study were obtained as lyophilized stocks from the Centraalbureau voor Schimmelcultures, Delft, The Netherlands (CBS 397, CBS 608, CBS 745 and CBS 7858) or the National Collection of Yeast Cultures Norwich, UK (NCYC 100, NCYC 179, NCYC 111, NCYC 587, NCYC 970, NCYC 1424, NCYC 2597, NCYC 2791, NCYC 2887). Some strains are also known by alternative names, notably, NCYC 2791 is also designated CBS 712 and is the type strain for the species, and NCYC 2597, which is the same strain as CBS 6556. The strains were isolated from diverse sources (mainly dairy) between 1925 and 1994 and are all reported to be Lac^{+} and $Inulinase^{+}$. The two *S. cerevisiae* strains used were the wild-type BY 4741 and the congenic *hxk2* mutant from the systematic gene deletion set (Winzeler et al. 1999). Yeast strains were routinely cultured at 30°C in YP medium (1% yeast extract; 2% bactopeptone) with 2% sugar (glucose/lactose/galactose as appropriate). On receipt from culture collections, strains were cultured in YPD and 1 ml stocks containing 15% glycerol were stored at −70°C.

Molecular identification and relatedness of *K. marxianus* strains

Strain identification and relatedness was ascertained by analysis of the rDNA locus. DNA was extracted from overnight cultures using a modified version of the Hoffman & Winston method and re-suspended in sterile distilled water (Hoffman and Winston 1987). The modifications involved extra vortexing and ethanol precipitation steps. The D1/D2 domain of the 26S rDNA gene was amplified using the primers NL-1 (5′-GCATATCAATAAGCGGAGGAA AAG) and NL-4 (5′-GGTCCGTGTTTCAAGACGG) (O'Donnell 1993) in a standard PCR reaction of annealing 30 s at 52°C, extension for 2 m at 72°C, and denaturation at 94°C for 1 m (36 cycles) (Kurtzman and Robnett 1998). The ITS spacer region of the rDNA was amplified using primers ITS-1 (5′-TCCGTAGGTGAACCTGCGG) and ITS-4 (5′- TCCTCCGCTTATTGATATGC) in a standard PCR reaction of annealing 1 m at 55°C, extension for 2 m at 72°C, and denaturation at 94°C for 1 m (35 cycles). PCR products were purified using the Qiagen QIAquick PCR microcentrifuge purification kit as per manufacturer's instructions and sequenced by GATC Germany. The strains were already classified as *K. marxianus* in the source strain databases so the main purpose was to confirm that no strain was in fact the close relative *Kluyveromyces lactis*. The sequence data was compared to references in databanks (CBS, NCYC, Genbank, NCBI) to confirm the identity of the 13 strains and their classification as *K. marxianus* (Supplementary Fig. 1).

Phenotypic assays

Serial dilutions of cultures onto agar plates were used to assess the tolerance of *K. marxianus* strains to a variety of stresses. For these assays, strains were grown overnight at 30°C to stationary phase in 10 ml YPL medium in a 20 ml sterilin tube with shaking. Yeast cells were diluted to A_{600} 0.2 in fresh YPL medium and serial dilutions down to 10^{-5} were prepared in 96 well microtitre plates and spotted aseptically onto YPL agar plates using a 48 pin replicator. To assess thermotolerance, plates were incubated at 24, 30, 37, 42, and 48°C but otherwise incubations were at 30°C. Various reagents were added to the medium to assess tolerance to salt (NaCl 0.5 M; 1 M), Calcofluor White (CFW) and caffeine (CAF). The CFW (F3543, Sigma) and CAF (C8960, Sigma) stock solutions were filter sterilised and added to autoclaved YPL agar cooled to 60°C at a range of final concentrations (0.1, 0.3, 0.5 mM CFW; and 10, 20, 50 mM CAF). The plates were incubated at 30°C in the dark and growth was recorded after 48 h. To assess glucose repression, strains were streaked onto YPGAL agar plates with or without the addition of 2-deoxy-D-glucose (Sigma) at a final concentration of 200 μg/ml from a 200 mg/ml stock. Plates were incubated for 48–60 h at 30°C.

Growth, ethanol and biomass production in *K. marxianus*

Exponential phase cultures of each strain were used to inoculate 50 ml YPL medium in 250 ml Erlenmeyer flasks to a final A_{600} of 0.2. The flasks were incubated at 30°C for ten hours with agitation at 180 rpm. Samples were collected at 1 h intervals and growth was recorded by absorbance at 600 nm. The maximum specific growth rate was determined from the slope of the curve during exponential growth. Supernatants were collected every 2 h and stored at −20°C for later determination of lactose and ethanol concentrations. Lactose and ethanol concentrations were determined by high performance liquid chromatography (HPLC) using an Agilent 1200 HPLC system with a refractive index detector. A REZEX 8 µl 8% H organic acid column 300 × 7.8 mM was used with 0.01 N H_2SO_4 elution fluid at a flow rate of 0.6 ml m^{-1}. The temperature of the column was maintained at 65°C. Substrate and end-product peaks were identified by comparison of their retention times with those of pure compounds and concentrations are determined from standards of known concentrations. To determine biomass after 10 h growth, 30 ml cultures were harvested by centrifugation at 5000 rpm × 10 m and washed twice with distilled water. Cells were dried at 100°C for 24 h and weighed to determine dry weigh. Independent triplicate cultures were always grown.

Analysis of the mating loci in *K. marxianus*

A genome sequence of *K. marxianus* CBS 608 was obtained by pyrosequencing using 454 technology. A draft assembly of the genome, assembled from 1.1 million Roche 454 Titanium FLX reads, yielded 246 scaffolds. This should deliver 26× genome coverage and assembly is ongoing. This draft assembly was interrogated to identify *HML*, *HMR* and *MAT* loci, based on BLAST with orthologous genes from *K. lactis*. Sequences of these loci have been lodged in European Nucleotide Archive with the following Accession Numbers: FR854193; FR854194; FR854195, and FR854196. The *MAT* locus is flanked by homologous genes in *K. lactis* and *K. marxianus* and this information was used to design consensus primers to the flanking *SLA2* and *LAA1* genes (should amplify *MAT* locus from all *K. lactis* and *K. marxianus* strains). Using these primers, FP1 (TATACATGGGATCATAAATC) and RP1 (CTTTGTCTTGTATGATATC), the *MAT* locus could be amplified from genomic DNA by PCR using the Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) enzyme under the following conditions: annealing 30 s at 46°C, extension for 9 m at 68°C, and denaturation at 94°C for 30 s (35 cycles).

Results

Analysis of mating type loci in *K. marxianus*

The 13 *K. marxianus* strains used in this study were chosen to provide a cross-section of isolates for physiological studies. They were sourced from two separate culture collections and included NCYC 2791 (CBS 712), which is the type strain for the species. The strains were originally isolated from different environments such as dairy, soil, plants, and fruit. The identity of all strains as *K. marxianus* was confirmed by sequencing the 26S D1/D2 region and the ITS spacer regions of the rDNA locus (Supplementary Fig. S1). Phylogenetic analysis of the rDNA and ITS sequences did not identify any sub-clades of strains among these 13 *K. marxianus* isolates (data not shown). As there was no complete genome sequence of any *K. marxianus* strain available, a genome sequence of strain CBS 608 was generated as a general resource. This strain was chosen because it possesses some traits that are of interest from a biotechnology perspective (described in subsequent sections below). From analysis of the draft genome assembly, it was possible to identify the mating type locus (Fig. 1a). The basic organization of the *MAT* locus is very similar to that of the sister species *K. lactis* with the *MAT**a*** and *MATα* genes flanked by orthologues of *SLA2* and *LAA1* (YJL207C) (Astrom and Rine 1998; Butler et al. 2004). The *K. marxianus* *MAT* loci are predicted to encode Mat**a**1, Mat**a**2, Matalpha1 and Matalpha2 proteins involved in regulating cell identity and mating, as well as the Matalpha3 protein recently shown to be involved in mating type switching in *K. lactis* (Barsoum et al. 2010). Despite the similar organization, the Mat proteins do show substantial divergence from the *K. lactis* orthologues at the amino acid level, ranging from 34% identity in Matalpha2 to 55% in Mat**a**2. Additional loci, predicted to be the silent *HMR**a*** and

HMLα loci were identified. As the homologues of the genes flanking the HML/HMR regions are located near telomeres in other species, it is likely that these loci are also telomeric in *K. marxianus*. The genes flanking *HML* and *HMR* in *K. marxianus* are mostly the same as in *K. lactis* (Dujon et al. 2004) with two exceptions. To the left of *HML* in *K. marxianus* (as drawn in Fig. 1a), there is an ortholog of *K. lactis* *KLLA0C18975* (*S. cerevisiae YKL215C*), which is a telomeric gene in *K. lactis* but not located beside *HML*. To the left of *HMR*, *K. lactis* has an extra gene located between *YHB1* and the duplicated 3′ end of *SLA2*. This extra gene, *KLLA0B14498g*, encodes a probable cell surface protein that appears not to have any orthologue in the *K. marxianus* genome.

The draft genome assembly indicated that CBS 608 is a diploid strain as both the *MAT**a*** and the *MATalpha* loci were identified flanked by full-length copies of *SLA2* and *LAA1* (Fig. 1a). DNA primers that anneal to the 5′ ends of the flanking *SLA2* and *LAA1* genes were designed to facilitate PCR amplification of the entire *MAT* locus. Using this primer set and genomic DNA, it was possible to establish the mating type and ploidy of the 13 *K. marxianus* strains under investigation in this study (Fig. 1b). Two bands of the expected size were amplified from CBS 608, consistent with the evidence that this strain is diploid. Three further strains, NCYC 100,111 and 179 are also diploid and the nine remaining strains are haploid. Of these, seven amplified a single band of a size consistent with the *MAT**a*** locus, and one NCYC 2597, amplified a band consistent with the *MATalpha* locus. CBS 745 is also haploid and appears to contain a deletion of approximately 1 kb at the *MAT* locus but this was not investigated any further in this study.

Stress tolerance of *K. marxianus* strains

The capacity to adapt to adverse environmental conditions is an important trait for industrial yeasts as industrial fermentation conditions may be sub-

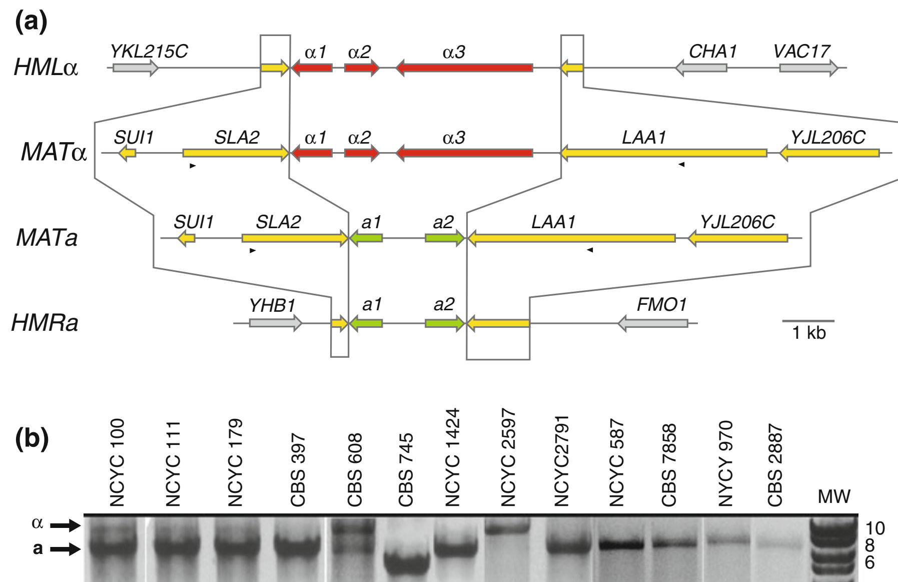

Fig. 1 Mating loci in *K. marxianus*. **a** Genome sequence from CBS 608 was used to reconstruct the *K. marxianus MAT, HML* and *HMR* regions. Genes are named according to their *S. cerevisiae* orthologues. Alpha-specific genes (online, *Red*); a-specific genes (online, *green*); flanking genes on the *MAT* chromosome (online, *yellow*) and genes flanking *HML* and *HMR* (online, *grey*) are shown. The *boxed areas* show the regions that are identical between the *MAT* and *HML/HMR* regions, including duplications of the 3′ ends of the genes *SLA2* and *LAA1*. The positions of DNA primers used in "**b**" are indicted with *arrows*. FP1 is located in *SLA2* and RP1 in *LAA1*. The sequences have been lodged in the European Nucleotide Archive with accession numbers FR854193; FR854194; FR854195 and FR854196. **b** DNA primers to amplify the entire *MAT* locus were designed from the CBS 608 sequence and used to amplify products from the 13 *K. marxianus* strains indicated. Based on the *K. marxianus* CBS 608 sequence, the predicted products was 9691 nt (*MATalpha*) or 6652 nt (*MAT**a***)

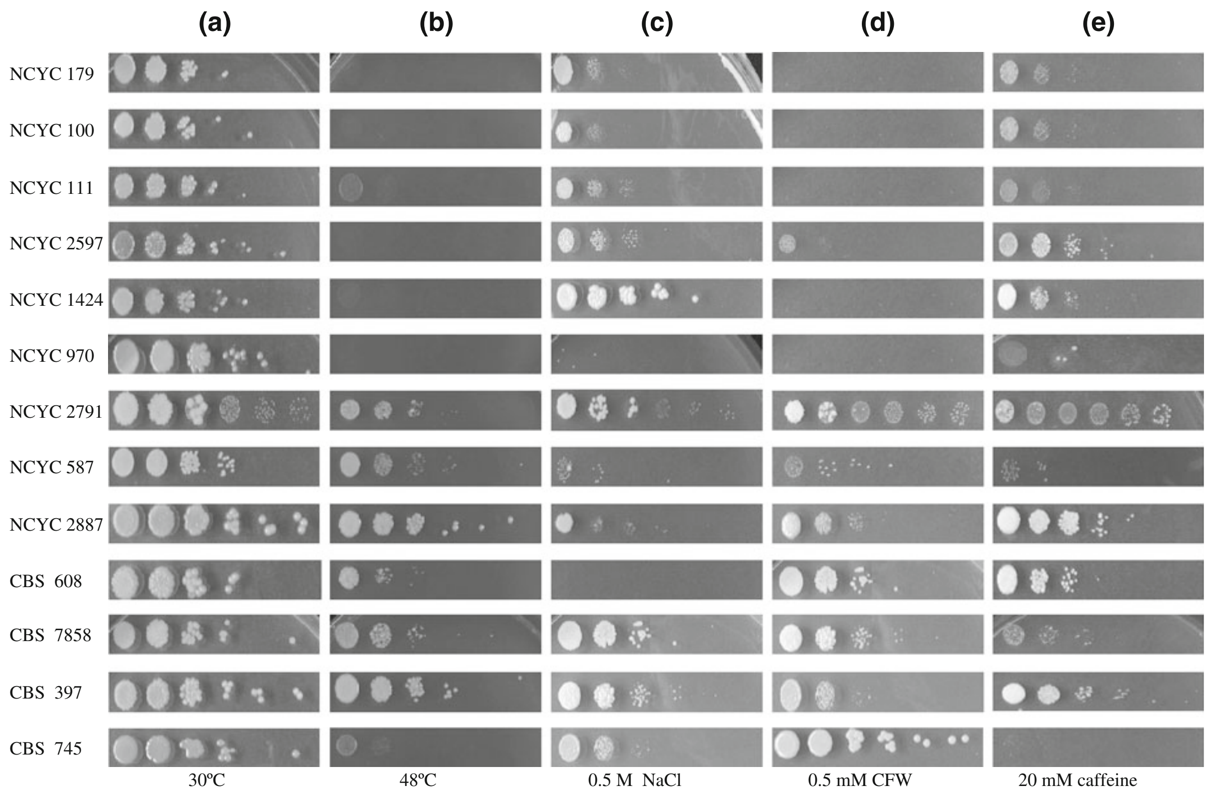

Fig. 2 Phenotypic diversity among *K. marxianus* strains in response to stress. The 13 *K. marxianus* strains indicated were pre-grown in YPL and serial dilutions were made in 96 well plates. These were aseptically replicated onto agar plates with YPL medium and the appropriate stress before being incubated at 30°C (other than the plates at 48°C, **b**). In each case several concentrations of the stressor were tested and the figure shows the most relevant conditions (see text for further details). **a** control plate, YPL, 30°C. **b** YPL medium, 48°C. **c** YPL medium, 0.5 M NaCl. **d** YPL, 0.5 mM CFW. **e** YPL, 20 mM caffeine)

optimal. The stress tolerance of the 13 *K. marxianus* strains was therefore compared (Fig. 2). First, the effect of elevated temperatures was assessed by testing growth at a range of temperatures from 24°C to 48°C. All strains showed similar growth at all temperatures up to 42°C, but at 48°C, seven strains failed to grow, four strains were strongly impaired and only two strains, CBS 397 and NCYC 2887, showed robust growth (Fig. 2b). Next, strains were exposed to 0.5 M NaCl to assess the capacity to resist salt stress (Fig. 2c). A range of sensitivities was evident, with some strains (e.g., NCYC 1424 and CBS 7858) quite tolerant, whereas others (e.g., NCYC 970) were very sensitive to this salt concentration. None of the strains grew on 1 M NaCl (data not shown). The third treatment was to expose cells to cell wall stress by treating with the anionic dye calcofluor white (CFW) (Fig. 2d). This molecule binds chitin and is believed to inhibit chitinase, leading to cell wall damage (Ram and Klis 2006). Most strains were sensitive to this cell wall damaging agent, but strains CBS 745 and NCYC 2791 were clearly more tolerant than other strains to 0.5 mM CFW. Finally, the *K. marxianus* strains were exposed to 20 mM caffeine (Fig. 2e). This drug is an adenosine analogue and has several targets in the cell in different signaling pathways (Hood-Degrenier 2011). It is typically linked to the response to cell wall damage because of its effects on the cell integrity pathway. A number of strains, notably CBS 397, 608 and NCYC 2887, were quite resistant to this drug, whereas others such as CBS 745 and NCYC 587 were highly sensitive. None of the strains grew on 50 mM caffeine (data not shown).

Table 1 Growth rate, biomass and ethanol production yield of *K. marxianus* during growth on YPL

Strain	Specific growth rate (μ) (h^{-1})	Biomass yield (dry weight) ($g\ g^{-1}$)	Ethanol yield ($g\ g^{-1}$)
NCYC 100	0.53	0.15	0.28
NCYC 179	0.63	0.14	0.28
NCYC 111	0.50	0.07	0.00
NCYC 2597	0.50	0.05	0.19
NCYC 1424	0.60	0.11	0.28
NCYC 970	0.6	0.11	0.32
NCYC 2791	0.63	0.07	0.03
NCYC 587	0.87	0.06	0.20
NCYC 2887	0.87	0.07	0.20
CBS 608	0.53	0.11	0.28
CBS 7858	0.87	0.06	0.32
CBS 397	0.60	0.15	0.31
CBS 745	0.99	0.06	0.15

Growth, biomass and ethanol production of 13 *K. marxianus* strains was recorded during fermentation of YPL in 250 ml Erlenmeyer flasks. Samples were collected at 1 h intervals and growth was recorded by optical density. Sample supernatants were stored at −20°C at every 2 h interval. Ethanol concentration was determined by HPLC analysis. After 10 h samples were taken and dried for 24 h at 100°C before recording dry weight. Averages of triplicate cultures are presented

Growth and fermentation characteristics of *K. marxianus* strains

Since one of the important applications of *K. marxianus* involves growth on whey permeate, the growth characteristics of the strains using lactose as the carbon source were determined. Strains were grown in shake flask culture in rich (YPL) medium and growth kinetics, biomass yield, and ethanol yield determined for each strain. These data are summarized in Table 1. Growth rates ranged from a μ_{max} of 0.50 h^{-1} for *K. marxianus* NCYC 111 to 0.99 h^{-1} for *K. marxianus* CBS 745. A large variation in biomass yield was also observed with a 3-fold difference between the strain with the highest (NCYC 100) and the lowest (NCYC 2597) yields. Most strains produced ethanol with yields of between 0.19 and 0.32 $g\ g^{-1}$ but one strain, CBS 745, was a lower producer and two strains, NCYC 2791 and NCYC 111 failed to produce appreciable amounts of ethanol. Detailed examination of the strains over the course of the 10 h time-course revealed several distinct patterns or growth and ethanol production (Fig. 3). Eleven of the 13 strains were classified as ethanol-producing but two distinct patterns of growth were apparent. Seven of the strains displayed the kinetics exemplified by CBS 397 (Fig. 3a) in which the strain grew exponentially until 8–10 h, when it entered stationary phase. The other four strains showed a very different pattern, exemplified by CBS 7858 (Fig. 3b), with rapid initial growth and entry into stationary phase after 4–5 h. For all producing strains, ethanol predominantly accumulated in stationary phase cultures after 8–10 h of growth, Different kinetics of lactose utilization were also seen, with seven of the strains (CBS 7845, NCYC 2791, NCYC 2579, NCYC 111, CBS 970, CBS 587, CBS 745) unable to completely deplete lactose from the growth medium. The two non-producing strains also showed unique patterns. NCYC 2791 (Fig. 3c) grew rapidly like CBS 7858 but did not fully utilization the lactose and only produced minimal levels of ethanol. The growth profile of NCYC 111 (Fig. 3d) was more similar to CBS 397 but lactose was incompletely utilized and no ethanol produced. As strain NCYC 111 was just entering stationary phase after 10 h growth, ethanol production was also assessed at later time-points. Even after 24 h incubation, only low levels of ethanol were generated (data not shown).

The capacity to ferment sugars to ethanol is very well-studied in *S. cerevisiae* and is linked to the phenomenon of carbon catabolite (glucose) repression in that yeast. To investigate whether glucose repression is present in *K. marxianus*, and whether it might correlate with the fermentative capacity of different strains, the 13 *K. marxianus* strains were grown in galactose-containing medium, either with or without the non-metabolisable glucose analogue, 2-deoxy glucose (2-DOG) (Fig. 4). As controls, wild-type *S. cerevisae* BY4741 and a congenic *S. cerevisiae hxk2* mutant, which lacks glucose repression were included. Very significant differences in the ability to grow in the presence of 2-DOG were observed. Five strains grew as well as the *S. cerevisiae hxk2* control (NCYC 111, 587, 1424, 2791, 2597) whereas eight strains displayed impaired or little growth (NCYC 100, NCYC 179, NCYC 970, NCYC 2887, CBS 608, CBS 397, CBS 745, CBS 7858). The extent of inhibition varied but other than noting that the two strains (NCYC 111 and NCYC

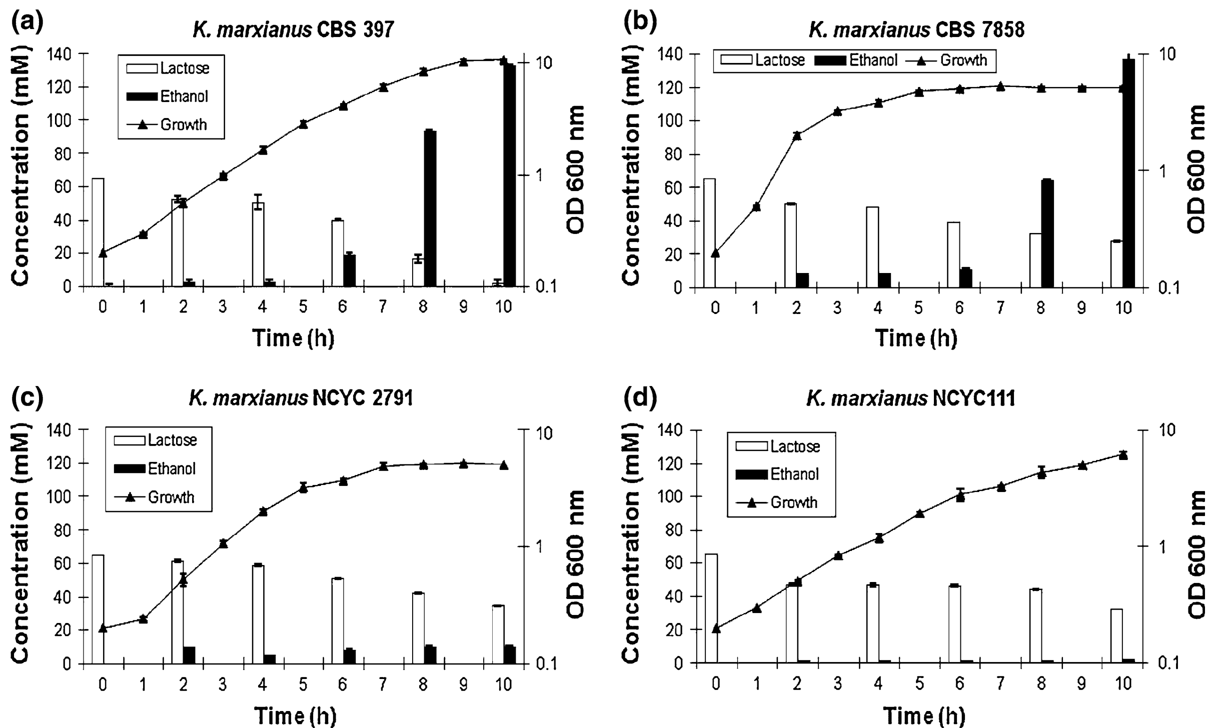

Fig. 3 Differential patterns of growth and lactose utilization among *K. marxianus* strains. Exponential phase cultures of *K. marxianus* grown in YPL were used to inoculate 50 ml YPL medium in 250 ml Erlenmeyer flasks to a final A_{600} of 0.2. Growth was followed by A_{600} over 10 h and supernatant samples were taken to measure ethanol and lactose levels. Thirteen strains were tested and the panels show strains representative of each pattern of growth/metabolism. **a** *K. marxianus* CBS 397, representing strains CBS 608, NCYC 100, 179, 970, 1424 and 2597. **b** *K. marxianus* CBS 7858 representing strains CBS 745, NCYC 587, 2887, 7858. **c** *K. marxianus* NYCY 2791. **d** *K. marxianus* NCYC 111. The mean values from triplicate cultures are presented with *error bars* indicating standard deviations (error bars not visible at some time-points)

2791) that failed to produce ethanol also showed no evidence of glucose repression, no particular correlations to the fermentation profiles (Fig. 3) were discernable.

Discussion

Kluyveromyces marxianus is generally described as lactose positive yeast commonly isolated from dairy environments, with traits such as rapid growth, inulinase production and thermotolerance (Fonseca et al. 2008; Lane and Morrissey 2010). Despite the diverse industrial applications to which the yeast is put, there is little systematic comparison of strains, nor of traits in the species. Genomic information is limited, with only a partial genome sequence of the type strain CBS 712/NCYC 2791 available (Llorente et al. 2000). As interest in the application of molecular genetics to this species grows, there is a need to gain a better understanding of the physiological and genetic variation within the species. The aim of this study was to focus on particular attributes that are important in industrial ethanol fermentations to ascertain the extent to which these traits were expressed in different isolates from two European culture collections.

The capacity to grow at elevated temperatures is one of the hallmarks of *K. marxianus* and this was evident in this study with all strains able to grow well at 42°C. At the higher temperature of 48°C, several strains continued to grow but only NCYC 2887 and CBS 397 showed strong growth. The physiological basis of high temperature growth is not known but based on work in *S. cerevisiae*; it is likely to be a polygenic trait (Cubillos et al. 2011). The

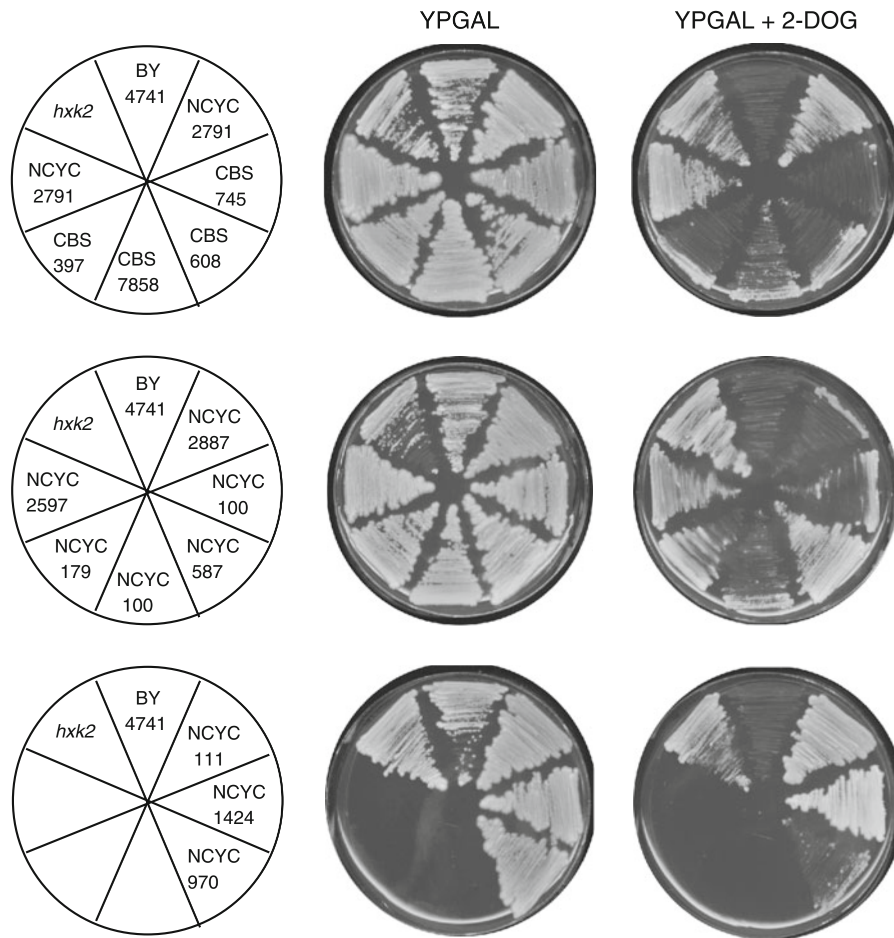

Fig. 4 Carbon catabolite repression in *K. marxianus*. *K. marxianus* strains were streaked onto YPGAL medium without (*center*) or with (*right*) with the glucose analogue 2-deoxy glucose (2-DOG). Growth on 2-DOG indicates a lack of glucose repression. Each plate also includes the wild type *S. cerevisiae* strain BY 4741 and the congenic *hxk2* mutant that lacks glucose repression. The left column indicates the strains (note that some strains are present in duplicate and the final plate contains just five strains)

phenomenon of diversity with respect to the maximum of temperature of growth illustrated in this study can also be seen with commercial isolates of *S. cerevisiae* (Salvado et al. 2011). Tolerance to extracellular stresses is also important in industrial yeasts and the diversity with respect to this was addressed by comparing osmotolerance and resistance to agents that trigger cell wall damage. The capacity to withstand an osmotic stress imposed by 0.5 M NaCl was highly variable among the isolates. Osmotolerance and osmoadaption is very well understood in *S. cerevisiae* and is primarily mediated by the high osmolarity/glycerol pathway (HOG), which can be activated by different membrane sensors (Hohmann 2009). The sensors and MAPK components are conserved across diverse yeast species and it is likely that a similar pathway may operate in *K. marxianus*. In support of this, recently, the *HOG1* gene was cloned and disrupted in a *K. marxianus* strain, resulting in a salt-sensitive phenotype (Qian et al. 2010). That study also showed that *HOG1* is required for tolerance to oxidative stress but found no link to thermotolerance. Our data are consistent with this, for while some strains such as CBS 397 are thermotolerant and osmotolerant, and others like NCYC 970 are sensitive to both high temperature and salt, more strains like NCYC 2887 and NCYC 1424 show opposite effects in response to the two stresses. Cell wall damaging agents also trigger an adaptive response in *S. cerevisiae*, this time via a different MAPK signal transduction pathway, the cell

wall integrity (CWI) pathway (Rodicio and Heinisch 2010; Rodriguez-Pena et al. 2010). This pathway is also important for resisting cell wall stress in *K. lactis* (Rodicio et al. 2008) and is therefore likely to be also present in *K. marxianus*. As with other stresses, a range of tolerance phenotypes to agents requiring the CWI pathway for an adaptive response was seen (calcofluor white, caffeine). The sensor protein Wsc1p has been linked to thermotolerance and the CWI pathway (Rodicio and Heinisch 2010), and it is notable that the more thermotolerant *K. marxianus* strains are also more tolerant to calcofluor white, but detailed molecular studies would be required to assess whether these phenotypes are linked in this species. The pattern of caffeine tolerance was similar to, but did not precisely overlap with, that of calcofluor white. For example, the most resistant strain to calcofluor white, CBS 745, was sensitive to caffeine. Although caffeine is often used to assess CWI function, it does have pleiotropic effects and it is likely that variation in another target is responsible for the enhanced sensitivity of CBS 745 (Hood-Degrenier 2011). In summary, the 13 strains displayed a wide variation in sensitivity to different environmental stresses consistent with the view that while *K. marxianus* does have stress response pathways, these are either defective or differently regulated in some strains. It is possible to identify strains, for example CBS 397 from this collection, that are tolerant to a range of stresses and such screens may provide the basis of identifying strains most suitable for industrial applications.

There have been extensive studies to understand the basis of the Crabtree effect in *S. cerevisiae* and the particular metabolism that leads to fermentative growth under aerobic conditions (Barnett and Entian 2005; Piskur et al. 2006). The general consensus is that it is a complex trait linked inter alia to sugar uptake kinetics, glycolytic flux and glucose repression. The unique metabolism of *S. cerevisiae* is certainly associated with the whole genome duplication that occurred approximately 100MYA, after the divergence of the *Saccharomyces* and *Kluyveromyces* genera (Wolfe and Shields 1997; Conant and Wolfe 2007). *K. lactis* and *K. marxianus* are designated Crabtree negative yeasts but, as with most of the *Saccharomycotina*, they have the potential to generate energy via respirofermentative metabolism, though some species are limited by an inability to recycle NADH (Merico et al. 2007). In *K. lactis,* the switch from respirative to fermentative growth seems to be controlled mainly by oxygen levels, with fermentation only occurring in response to oxygen limitation (Merico et al. 2009). The situation with *K. marxianus* is less clear, with many reports of different strains used to generate ethanol by fermentation in industrial processes (Fonseca et al. 2008; Lane and Morrissey 2010). To try to get a better understanding, we assessed our 13 *K. marxianus* strains for ethanol production in shake flask culture and observed clear phenotypic differences. All strains that produced ethanol did so mainly after 8–10 h, which would be consistent with an oxygen trigger as limitation does occur even in shake flasks. Nonetheless, although this may explain some strains such as CBS 7858, which only produce ethanol after several hours in stationary phase, it is a less satisfactory explanation for strains like CBS 397, where ethanol production seems to follow lactose depletion more closely. Furthermore, when grown in a chemostat with constant aeration, CBS 397 produced comparable amounts of ethanol, suggesting that oxygen limitation is less relevant in this strain (data not shown). These observations would need to be followed up with detailed studies where precise oxygen levels are measured during growth to determine the potential link between oxygen limitation and ethanol generation. Since oxygen depletion is related to growth rate, it is also likely that altering nutritional and environmental conditions may differentially affect the kinetics of ethanol generation in particular strains of *K. marxianus*. Some strains such as NCYC 2791 and NCYC 111 did not produce appreciable ethanol even after extended incubation in stationary phase, further emphasizing the metabolic differences between the strains. The ethanol yields obtained in this study are lower than that which can be obtained by *S. cerevisiae* (growing in glucose) but it should be noted that the studied strains were not specifically selected on the basis of ethanol production. The ethanol yields reported here are comparable to another recent study reporting the production of bioethanol from whey (Christensen et al. 2011). Furthermore, one recent study that did attempt to identify high ethanol producers generated a mutant *K. marxianus* strain that has comparable ethanol yields to *S. cerevisae,* indicating that the potential for efficient ethanol production by fermentation is present in *K. marxianus* (Pang et al. 2010). It is known

that carbon catabolite repression is important for the Crabtree effect in *S. cerevisae* as genes encoding TCA cycle enzymes are repressed by glucose via the Hxk2p/Snf1p/Mig1p system. Although *Kluyveromyces* species are reported to lack glucose repression, there was very clear variation among the strains tested in this study. As there appeared to be a continuum from strong to absent glucose repression, it is relevant to highlight a recent study with *S. cerevisiae,* which reported that genetic differences between strains gives rise to differential glucose repression in that yeast (Kummel et al. 2010). Patterns of glucose repression in *K. marxianus* did not correlate with fermentation traits, indicating that these are independent phenotypes. It was notable that the rate of lactose utilization varied among the strains, perhaps indicating that differences in lactose uptake kinetics and metabolic flux could be an important variable. It would be interesting in future studies to establish the copy number and expression levels of the *LAC12* permease in strains of *K. marxianus* to determine correlations with lactose utilization and ethanol generation. In addition, the possibility of a Kluyver effect should be investigated. This effect, which is manifest as the inability to transport specific sugars in the absence of oxygen, is known to occur for some sugars in *K. lactis* (Fukuhara, 2003) and could possibly explain the poor lactose utilization by some *K. marxianus* strains. Alternatively, another limiting factor in YP medium, such as absence of a required vitamin or cofactor or inadequate levels of a metabolic building block may restrict growth of these strains.

The major conclusion from this study is that *K. marxianus* is a phenotypically diverse species and broad generalizations about its traits may not be accurate. The divergent phenotypes among isolates provide a resource to attempt to dissect the precise pathways involved in various environmental stress responses. Understanding these responses and pathways will facilitate selection of robust strains and may allow reverse engineering of traits into *K. marxianus* or other industrial yeasts. Molecular analysis of ploidy in the species supports the emerging view that, unlike *K. lactis*, both haploid and diploid strains occur stably in natural environments. This potentially offers another route to developing new strains via mating and selection. The general picture of *K. marxianus* is as a species that is substantially different to the related *K. lactis* and one that promises more surprises when molecular tools are used to dissect in detail some of the traits discussed in this study.

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References

Astrom SU, Rine J (1998) Theme and variation among silencing proteins in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Genetics 148(3):1021–1029

Barnett JA, Entian KD (2005) A history of research on yeasts 9: regulation of sugar metabolism. Yeast 22(11):835–894

Barsoum E, Martinez P, Astrom SU (2010) Alpha 3, a transposable element that promoters host sexual reproduction. Genes Dev 24:33–44

Blank LM, Lehmbeck F, Sauer U (2005) Metabolic-flux and network analysis in fourteen hemiascomycetous yeasts. FEMS Yeast Res 5(6–7):545–558

Butler G, Kenny C, Fagan A, Kurischko C, Gaillardin C, Wolfe KH (2004) Evolution of the *MAT locus* and its Ho endonuclease in yeast species. Proc Natl Acad Sci USA 101(6):1632–1637

Christensen AD, Kadar Z, Oleskowicz-Popiel P, Thomsen MH (2011) Production of bioethanol from organic whey using *Kluyveromyces marxianus*. J Ind Microbiol Biotechnol 38(2):283–289

Conant GC, Wolfe KH (2007) Increased glycolytic flux as an outcome of whole-genome duplication in yeast. Mol Syst Biol 3:129

Cubillos FA, Billi E, Zorgo E, Parts L, Fargier P, Omholt S, Blomberg A, Warringer J, Louis EJ, Liti G (2011) Assessing the complex architecture of polygenic traits in diverged yeast populations. Mol Ecol 20(7):1401–1413

Dujon B, Sherman D, Fischer G, Durrens P, Casaregola S, Lafontaine I, De Montigny J, Marck C, Neuveglise C, Talla E, Goffard N, Frangeul L, Aigle M, Anthouard V, Babour A, Barbe V, Barnay S, Blanchin S, Beckerich JM, Beyne E, Bleykasten C, Boisrame A, Boyer J, Cattolico L, Confanioleri F, De Daruvar A, Despons L, Fabre E, Fairhead C, Ferry-Dumazet H, Groppi A, Hantraye F, Hennequin C, Jauniaux N, Joyet P, Kachouri R, Kerrest A, Koszul R, Lemaire M, Lesur I, Ma L, Muller H, Nicaud JM, Nikolski M, Oztas S, Ozier-Kalogeropoulos O, Pellenz S, Potier S, Richard GF, Straub ML, Suleau A, Swennen D, Tekaia F, Wesolowski-Louvel M, Westhof E,

Wirth B, Zeniou-Meyer M, Zivanovic I, Bolotin-Fukuhara M, Thierry A, Bouchier C, Caudron B, Scarpelli C, Gaillardin C, Weissenbach J, Wincker P, Souciet JL (2004) Genome evolution in yeasts. Nature 430(6995):35–44

Fonseca GG, Heinzle E, Wittmann C, Gombert AK (2008) The yeast *Kluyveromyces marxianus* and its biotechnological potential. Appl Microbiol Biotechnol 79(3):339–354

Fukuhara H (2003) The Kluyver effect revisited. FEMS Yeast Res 3(4):327–331

Fukuhara H (2006) *Kluyveromyces lactis*—a retrospective. FEMS Yeast Res 6(3):323–324

Hoffman CS, Winston F (1987) A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene 57(2–3): 267–272

Hohmann S (2002) Osmotic stress signaling and osmoadaptation in yeasts. Microbiol Mol Biol Rev 66(2):300–372

Hohmann S (2009) Control of high osmolarity signalling in the yeast *Saccharomyces cerevisiae*. FEBS Lett 583(24):4025–4029

Hohmann S, Krantz M, Nordlander B (2007) Yeast osmoregulation. Methods Enzymol 428:29–45

Hood-Degrenier JK (2011) Identification of phosphatase 2A-like Sit4-mediated signalling and ubiquitin-dependent protein sorting as modulators of caffeine sensitivity in *S. cerevisiae*. Yeast 28(3):189–204. doi:10.1002/yea.1830

Kawasaki L, Castaneda-Bueno M, Sanchez-Paredes E, Velazquez-Zavala N, Torres-Quiroz F, Ongay-Larios L, Coria R (2008) Protein kinases involved in mating and osmotic stress in the yeast *Kluyveromyces lactis*. Eukaryot Cell 7(1):78–85

Kummel A, Ewald JC, Fendt SM, Jol SJ, Picotti P, Aebersold R, Sauer U, Zamboni N, Heinemann M (2010) Differential glucose repression in common yeast strains in response to *HXK2* deletion. FEMS Yeast Res 10(3):322–332

Kurtzman CP (2003) Phylogenetic circumscription of *Saccharomyces, Kluyveromyces* and other members of the *Saccharomycetaceae*, and the proposal of the new genera *Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma* and *Zygotorulaspora*. FEMS Yeast Res 4(3):233–245

Kurtzman CP, Robnett CJ (1998) Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. Antonie Van Leeuwenhoek 73(4):331–371

Lachance MA (2007) Current status of *Kluyveromyces* systematics. FEMS Yeast Res 7(5):642–645

Lane MM, Morrissey JP (2010) *Kluyveromyces marxianu*s: a yeast emerging from its sister's shadow. Fungal Biol Rev 24:17–26

Li H, Johnson AD (2010) Evolution of transcription networks–lessons from yeasts. Curr Biol 20(17):R746–R753

Llorente B, Malpertuy A, Blandin G, Artiguenave F, Wincker P, Dujon B (2000) Genomic exploration of the hemiascomycetous yeasts: 12 *Kluyveromyces marxianus* var. *marxianus*. FEBS Lett 487(1):71–75

Merico A, Sulo P, Piskur J, Compagno C (2007) Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. FEBS J 274(4):976–989

Merico A, Galafassi S, Piskur J, Compagno C (2009) The oxygen level determines the fermentation pattern in *Kluyveromyces lactis*. FEMS Yeast Res 9(5):749–756

O'Donnell K (1993) Fusarium and its near relatives. In: Reynolds RR, Taylor JWW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematic. CAB International, Wallingford, UK

Pang ZW, Liang JJ, Qin XJ, Wang JR, Feng JX, Huang RB (2010) Multiple induced mutagenesis for improvement of ethanol production by *Kluyveromyces marxianus*. Biotechnol Lett 32(12):1847–1851

Piskur J, Rozpedowska E, Polakova S, Merico A, Compagno C (2006) How did *Saccharomyces* evolve to become a good brewer? Trends Genet 22(4):183–186

Porro D, Branduardi P (2009) Yeast cell factory: fishing for the best one or engineering it? Microb Cell Fact 8:51

Qian J, Qin X, Yin Q, Chu J, Wang Y (2010) Cloning and characterization of *Kluyveromyces marxianus HOG1* gene. Biotechnol Lett 33(3):571–575

Ram AF, Klis FM (2006) Identification of fungal cell wall mutants using susceptibility assays based on Calcofluor white and Congo red. Nat Protoc 1(5):2253–2256

Rodicio R, Heinisch JJ (2010) Together we are strong–cell wall integrity sensors in yeasts. Yeast 27(8):531–540

Rodicio R, Buchwald U, Schmitz HP, Heinisch JJ (2008) Dissecting sensor functions in cell wall integrity signaling in *Kluyveromyces lactis*. Fungal Genet Biol 45(4):422–435

Rodriguez-Pena JM, Garcia R, Nombela C, Arroyo J (2010) The high-osmolarity glycerol (HOG) and cell wall integrity (CWI) signalling pathways interplay: a yeast dialogue between MAPK routes. Yeast 27(8):495–502

Rozpedowska E, Galafassi S, Johansson L, Hagman A, Piskur J, Compagno C (2011) *Candida albicans*—a pre-whole genome duplication yeast— is predominantly aerobic and a poor ethanol producer. FEMS Yeast Res 11(3):285–291

Rubio-Texeira M (2006) Endless versatility in the biotechnological applications of *Kluyveromyces LAC* genes. Biotechnol Adv 24(2):212–225

Salvado Z, Arroyo-Lopez FN, Guillamon JM, Salazar G, Querol A, Barrio E (2011) Temperature adaptation markedly determines evolution within the *Saccharomyces* genus. Appl Environ Microbiol 77(7):2292–2302

Schaffrath R, Breunig KD (2000) Genetics and molecular physiology of the yeast *Kluyveromyces lactis*. Fungal Genet Biol 30(3):173–190

Smith DA, Morgan BA, Quinn J (2010) Stress signalling to fungal stress-activated protein kinase pathways. FEMS Microbiol Lett 306(1):1–8

Suleau A, Jacques N, Reitz-Ausseur J, Casaregola S (2005) Intraspecific gene expression variability in the yeast *Kluyveromyces lactis* revealed by micro-array analysis. FEMS Yeast Res 5(6–7):595–604

Winzeler EA, Shoemaker DD, Astromoff A, Liang H, Anderson K, Andre B, Bangham R, Benito R, Boeke JD, Bussey H, Chu AM, Connelly C, Davis K, Dietrich F, Dow SW, El Bakkoury M, Foury F, Friend SH, Gentalen E, Giaever G, Hegemann JH, Jones T, Laub M, Liao H, Liebundguth N, Lockhart DJ, Lucau-Danila A, Lussier M, M'Rabet N, Menard P, Mittmann M, Pai C, Rebischung C, Revuelta

JL, Riles L, Roberts CJ, Ross-MacDonald P, Scherens B, Snyder M, Sookhai-Mahadeo S, Storms RK, Veronneau S, Voet M, Volckaert G, Ward TR, Wysocki R, Yen GS, Yu K, Zimmermann K, Philippsen P, Johnston M, Davis RW (1999) Functional characterization of the *S cerevisiae* genome by gene deletion and parallel analysis. Science 285(5429):901–906

Wolfe KH, Shields DC (1997) Molecular evidence for an ancient duplication of the entire yeast genome. Nature 387(6634):708–713