MINIREVIEW

Pichia stipitis genomics, transcriptomics, and gene clusters

Thomas W. Jeffries1,2 & Jennifer R. Headman Van Vleet2

1 USDA Forest Products Laboratory, Madison, WI, USA and 2 Department of Bacteriology, University of Wisconsin-Madison, Madison, WI, USA

Correspondence: Thomas W. Jeffries, Institute for Microbial and Biochemical Science, 1 Gifford Pinchot Drive, Madison, WI 53705, USA. Tel.: +1 608 231 9453; fax: +1 608 231 9262; e-mail: twjeffri@wisc.edu

Received 30 January 2009; revised 16 April 2009; accepted 17 April 2009. Final version published online 29 July 2009.

DOI:10.1111/j.1567-1364.2009.00525.x

Editor: Jens Nielsen

Keywords
yeast; evolution; genome; tandem repeats; orthologs; expression arrays.

Abstract

Genome sequencing and subsequent global gene expression studies have advanced our understanding of the lignocellulose-fermenting yeast Pichia stipitis. These studies have provided an insight into its central carbon metabolism, and analysis of its genome has revealed numerous functional gene clusters and tandem repeats. Specialized physiological traits are often the result of several gene products acting together. When coinheritance is necessary for the overall physiological function, recombination and selection favor colocation of these genes in a cluster. These are particularly evident in strongly conserved and idiomatic traits. In some cases, the functional clusters consist of multiple gene families. Phylogenetic analyses of the members in each family show that once formed, functional clusters undergo duplication and differentiation. Genome-wide expression analysis reveals that regulatory patterns of clusters are similar after they have duplicated and that the expression profiles evolve along with functional differentiation of the clusters. Orthologous gene families appear to arise through tandem gene duplication, followed by differentiation in the regulatory and coding regions of the gene. Genome-wide expression analysis combined with cross-species comparisons of functional gene clusters should reveal many more aspects of eukaryotic physiology.

Introduction

Pichia stipitis has a set of physiological traits that make it very useful for the bioconversion of lignocellulose. In addition to its extensively studied capacity for xylose fermentation, it is also able to ferment, glucose, mannose, galactose and cellobiose along with mannans and xylan oligomers. This makes it a potent organism for hydrolysate or simultaneous saccharification and fermentation (SSF). After glucose, xylose is the second most abundant hemicellulosic component in agricultural residues and fast-growing hardwood species, and cellobiose is the primary sugar formed in enzymatic hydrolysis.

Hemicellulosic sugars are readily recovered following treatment with alkali, dilute acid or autohydrolysis; hence, their use is critical to economical bioconversion. The capacity of P. stipitis to use cellobiose is important in SSF because commercial cellulase preparations are often deficient in β-glucosidase and the accumulation of cellobiose inhibits cellulase activities. The capacity of P. stipitis to use oligomeric sugars is important because mild acid pretreatments can avoid the formation of sugar degradation products, but can leave 15–50% of the solubilized hemicellulose in an oligomeric form.

Hemicellulosic sugars are underutilized even though they can be readily recovered with higher yields and at lower cost than glucose from cellulose. While they can be converted into a number of useful products such as xylitol, lactic acid and other chemicals, the product with the largest potential market is ethanol. Production of ethanol from lignocellulosic sources will continue to increase as a consequence of energy and agriculture policy incentives to create renewable alternative fuels and reduce carbon dioxide emissions.

Researchers in numerous laboratories have borrowed genes from P. stipitis and other fermentative microorganisms to modify Saccharomyces cerevisiae for xylose, xylan or cellulose metabolism. While partly successful, efficient xylose utilization has been impaired by S. cerevisiae’s generally low rate of xylose consumption and its inappropriate regulatory responses. It lacks sufficient levels of the assimilatory genes, sugar transporters and mechanisms for balancing cofactor levels under oxygen-limiting conditions.

We understand most of the biochemical steps required for xylose utilization, but a more thorough knowledge of
how native xylose-fermenting yeasts such as *P. stipitis* regulate their metabolism could advance the field on several fronts. Recent genomic and transcriptomic studies have revealed the presence of functional gene clusters that mediate the use of lignocellulosic substrates. This review briefly examines the physiological and fermentative properties of *P. stipitis*, and focuses on its genomic and regulatory features.

**A novel yeast for lignocellulose bioconversion**

Rational metabolic engineering requires a detailed understanding of physiology, biochemistry and genetics. This is possible only when the major pathways and mechanisms are known. Biochemical and genetic characterization of xylose fermentation by *P. stipitis* Pignall (1967) (*Yamadazyma stipitis*) has been underway for at least 15 years since the development of systems for its genetic transformation (Yang et al., 1994; Lu et al., 1998a; Laplaza et al., 2006) and mating (Melake et al., 1996). Relatively few researchers, however, have attempted its rational modification despite the fact that native strains produce more ethanol from xylose than any other studied yeast – including genetically modified *S. cerevisiae*.

*Pichia stipitis* has the highest native capacity for xylose fermentation of any known microorganism (van Dijken et al., 1986; du Preez et al., 1989). This yeast was originally isolated from insect larvae and is closely related to several yeast endosymbionts of passalid beetles (Nardi et al., 2006) that inhabit and degrade white-rotted hardwood (Suh et al., 2003). It is a predominantly haploid, homothallic, hemiascomycetous yeast (Vaughan Martini, 1984; Kurtzman, 1990; Gupthar, 1994; Melake et al., 1996) that forms buds along with pseudomycelia during vegetative growth, and two hat-shaped ascospores from each ascus. Fed batch *P. stipitis* cultures produce up to 47 g L\(^{-1}\) of ethanol from xylose at 30 °C (du Preez et al., 1989) with ethanol yields of 0.35–0.44 g g\(^{-1}\) xylose (Hahn-Hägerdal & Pamment, 2004), and they are capable of fermenting sugars from hemicellulosic acid hydrolysates with a yield equivalent to about 80% of the maximum theoretical conversion efficiency (Nigam, 2001a,b).

The genome of *P. stipitis* codes for cellulases, mannases, xylanase and other degradative enzymes that enable survival and growth in a wood-inhabiting, insect-gut environment (Nardi et al., 2006). *Pichia stipitis* has the capacity to ferment xylose, xylan (Lee et al., 1986; Ozcan et al., 1991) and cellobiose, and to use all of the major sugars found in wood, including arabinose and rhamnose (Koivistoinen et al., 2008). For these reasons, *P. stipitis* has been a common source of genes for engineering xylose metabolism in *S. cerevisiae* (Jeffries & Jin, 2004).

*Pichia stipitis* also has a number of other bioconversion-related traits: it modifies low-molecular-weight lignin moieties (Targonski, 1992), reduces acyclic enones to the corresponding alcohols (Conceicao et al., 2003), forms various esters and aroma components (Fuganti et al., 1993) and can be engineered to produce lactic acid (Ilmen et al., 2007) or xylitol (Kim et al., 2001; Rodrigues et al., 2008) in high yield. Strains of *P. stipitis* have also been selected for resistance to furfural and hydroxy-methyl furfural (Liu et al., 2005).

Metabolic engineering and adaptive evolution of *S. cerevisiae* for xylose fermentation has been successful to varying degrees (Harhangi et al., 2003; Sonderegger et al., 2004; Karhumaa et al., 2005). Engineering it with the basic assimilatory machinery of *XYL1, XYL2, XYL3* (or *XKS1*), *TAL1, TKL1, RPE1* and *RPI1* enables ethanol production. Expressing xylose isomerase (Wiedemann et al., 2006; van Maris et al., 2007; Wiedemann & Boles, 2008) or xylose reductases and xylitol dehydrogenases with altered cofactor specificities (Matsushika et al., 2008; Petschacher & Nidetzky, 2008) reduces cofactor imbalances, and increases the ethanol yield. It is not yet clear as to which of these engineering approaches will prove to be more successful in *S. cerevisiae* (Karhumaa et al., 2007).

Overexpression of *P. stipitis* or other fungal sugar transporters can also improve the performance of engineered *S. cerevisiae* on xylose (Weierstall et al., 1999; Saloheimo et al., 2007; Hector et al., 2008; Katahira et al., 2008; Leandro et al., 2008), but additional regulatory engineering is necessary because *S. cerevisiae* does not possess mechanisms to coordinate ethanol production in response to xylose (Jin et al., 2004). Therefore, even though the genetic tools, detailed biochemical knowledge and physiological properties of *S. cerevisiae* hold great promise for engineering the fermentation of xylose, xylan, cellulose, arabinose, rhamnose (Koivistoinen et al., 2008) and other sugars, much remains to be learned from *P. stipitis* and other yeasts that use these substrates natively. Conversely, the mechanisms *S. cerevisiae* use to ferment xylose can be adapted to improve the performance of *P. stipitis*.

*Pichia stipitis* shunts most of its metabolic flux into ethanol, and produces very little xylitol, but its fermentation rate on xylose is low relative to that of *S. cerevisiae* on glucose. Glucose and xylose are not equivalent fermentations for many reasons, but increasing the capacity of *P. stipitis* for rapid xylose fermentation could greatly improve its usefulness in commercial applications. Unlike *S. cerevisiae*, which regulates fermentation by sensing the presence of glucose, *P. stipitis* induces fermentative activity in response to oxygen limitation (Passoth et al., 1996, 2003a,b; Klinner et al., 2005). This, however, does not constitute the only fermentative regulatory mechanism. Global expression array analysis has shown specific response
patterns for xylose, cellobiose, arabinose, rhamnose and other lignocellulosic substrates. It is not fully known whether these are attributable to carbon catabolite derepression or specific induction. Our expression array results show evidence for both.

**Physiological features of *P. stipitis***

Most of the research with *P. stipitis* has focused on its capacity to ferment xylose. Even so, relatively little has been established concerning the rate-limiting steps in ethanol production from this sugar. In an early work, Bicho et al. (1988) showed that xylose reductase (Xyl1) and xylitol dehydrogenase (Xyl2) are repressed by glucose and induced during growth on xylose. Xylose is generally not consumed in the presence of glucose; hence, under glucose repression, these activities, along with xylose transport, are rate limiting or specific induction. Our expression array results show evidence for both.

Increasing the expression of *XYL1* for xylose reductase (Takuma et al., 1991) increased the enzymatic activity almost twofold, but had no beneficial effect on ethanol production (Dahn et al., 1996). To date, the overexpression of *XYL2* in *P. stipitis* has not been examined (Kotter et al., 1990); however, deletion of *XYL2* blocks xylose utilization at the level of xylitol and prevents its growth on this carbon source (Shi et al., 2000; Kim et al., 2001; Laplaza et al., 2006).

The respiratory capacity of *P. stipitis* is noted. In a respiration-limited, growth condition, oxidative phosphorylation provides energy for most, if not all, cellular activities. The latter has been cloned and characterized from *P. stipitis* NRRL Y-11543 (Basaran et al., 2001). The published xylanases sequence does not match with any identified ORF in the sequenced genome of *P. stipitis* CBS 6054 (= NRRL Y-11545, ATCC 58785), but the sequenced genome does include Family 10 endo-1,4-β-xylanase, and endoglucanase activities that might also act on xylan.

*Pichia stipitis* possesses β-xylosidase (Manzanares et al., 1999; Basaran & Özcan, 2008) and native Family 11 xylanase activities. The latter has been cloned and characterized from *P. stipitis* NRRL Y-11543 (Basaran et al., 2001). The published xylanases sequence does not match with any identified ORF in the sequenced genome of *P. stipitis* CBS 6054 (= NRRL Y-11545, ATCC 58785), but the sequenced genome does include Family 10 endo-1,4-β-xylanase, and endoglucanase activities that might also act on xylan. *Pichia stipitis*’s native xylanase activity has been supplemented through heterologous expression (Passoth & Hahn-Hagerdal, 2000; Gorgens et al., 2005).

**Genetic tools***

Genetic manipulation of *P. stipitis* is more difficult than with *S. cerevisiae* for multiple reasons. First, there are relatively few useful transformable *P. stipitis* host strains. Second, *P. stipitis* is resistant to most common antibiotics. Third, it uses an alternative codon system that substitutes serine for leucine when a CUG is encountered, which means that expression of foreign proteins, including those used as drug resistance markers, frequently requires some codon modification. Lastly, random (nonhomologous) integration is far more frequent than site-specific integration, which makes targeted deletions much harder to obtain. With these difficulties, however, researchers have made progress in developing genetic tools.

Melake et al. (1996) first developed a sporulation and mating system for this yeast; Yang et al. (1994) developed a genetic transformation system based on the *ura3* selectable marker; Lu et al. (1998a) expanded this to *leu2*; and Piontek et al. (1998) developed *trp5* and *his3* as auxotrophic markers in combination with a heterologous autonomous replication sequence. Laplaza et al. (2006) developed the first useful drug resistance marker, Sh ble (zeocin), and the loxp/Cre excision system by modifying the CUG codons in these proteins (Sugita & Nakase, 1999). Deletion of the *KU80* gene that is responsible for nonhomologous end joining significantly increases the fraction of homologous recombinant transformants, albeit at the expense of transformation.
frequency (Maassen et al., 2008). Other yeast-based transformation vectors are also useful with \textit{P. stipitis} (Klabunde et al., 2003). With the publication of the \textit{P. stipitis} genome, interest in and genetic tools for this organism have been steadily growing. A summary of strain development and genetic tools useful for \textit{P. stipitis} has been published recently (Jeffries, 2008).

**The \textit{P. stipitis} genome**

With sequencing and annotation of its genome (Jeffries et al., 2007), many diverse elements of the physiology of this yeast have come to light. Space permits only a brief summation of genes that can be found in their most complete annotations on the Joint Genome Institute web site and in other formats on the NCBI, Genamics and KEGG web sites. This publication constitutes a review of those published features. In addition to its original sequence, the SHI-21 \textit{cyc1} mutant of \textit{P. stipitis} CBS 6054 has been resequenced (Smith et al., 2008), revealing a total of 14 point mutations accumulated through mutation and selection over a period of about 7 years.

Sequencing showed that \textit{P. stipitis} CBS 6054 possesses eight chromosomes, of which two pairs are very similar in size, accounting for the earlier results suggesting the presence of six chromosomes (Passoth et al., 1992). Synteny analysis with its nearest completely sequenced yeast genome neighbor, \textit{Debaryomyces hansenii}, showed extensive recombination and shuffling of the chromosomes, which appears to be a common feature.

Sequencing the \textit{P. stipitis} genome has yielded numerous gene targets for metabolic engineering. In addition to the basic pathway for xylose, arabinose, mannose, galactose and rhamnose (Koivistoinen et al., 2008) metabolism, the genome has a number of sugar transporters (Weierstall et al., 2008), \(\beta\)-glucosidases, endoglucanases and a slew of NADH and NADPH-linked alcohol dehydrogenases, all of which could contribute to the fermentative capacities of the host.

**Expression array studies**

Genomic sequences alone do not reveal much about the function of a gene unless it is highly conserved and its function is known in other organisms. While the structures of genes coding for many core functions are highly similar to those found in \textit{S. cerevisiae} and other fungi, genes coding for idiomatic functions are notably different. Expression arrays have proved vital in understanding the functions of \textit{P. stipitis} genes. Moreover, multiple homologs often have different expression patterns indicative of different functions.

Consistent culture conditions are very important in obtaining data that can be compared across different experiments. All cultivations for expression arrays described here were performed in New Brunswick Scientific Bioflo 110 3-L bioreactors with working volumes of 2 L each. The bioreactors were equipped with two impellers rotating at 750 r.p.m. The bioreactor temperature was controlled at 30 °C and the pH was kept constant at 5.0 by automatic addition of 5 N KOH. Airflow into the bioreactors for aerobic cultivations was 1 v.v.m. \textit{Pichia stipitis} CBS 6054 was cultivated aerobically as described above and an aerobic transcriptomics sample was obtained during the exponential growth phase. Following sample collection, air and nitrogen were mixed to an initial oxygen concentration of 2% \(O_2\) using a Matheson gas proportioner, and the bioreactors were sparged at a rate of 0.5 v.v.m. Cultures were grown under low oxygen for 10–12 h, and a second, low-oxygen transcriptomics sample was obtained. Cultivations and sample collections for each carbon source were performed in triplicate.

A defined minimal medium containing trace metal elements and vitamins in the following amounts was used in all bioreactor cultivations: 2.4 g urea L\(^{-1}\), 3 g KH\(_2\)PO\(_4\) L\(^{-1}\), 0.5 g MgSO\(_4\) \(7H_2O\) L\(^{-1}\), 1 mL trace element solution L\(^{-1}\), 1 mL vitamin solution L\(^{-1}\) and 0.05 mL antifoam 289 L\(^{-1}\) (Sigma A-8436)(modified from Verduyn et al., 1992). For glucose, xylose or arabinose cultivations, a starting concentration of 50 g L\(^{-1}\) of sugar was used. For cellobiose cultivations, a starting concentration of 30 g L\(^{-1}\) of cellobiose was used.

Transcriptomics samples were centrifuged for 5 min at 4500 g in an Eppendorf 5804R centrifuge equipped with an A-4-44 rotor. The supernatant solutions were removed, and the cell pellets were flash frozen in liquid nitrogen. Cell pellets were stored at \(-80\) °C before RNA extraction. RNA was extracted using the Qiagen RNEasy Maxi kit (Qiagen). Cell breakage was accomplished using glass beads in a BeadBeater (Biospec Products) equipped with a 15-mL chamber. RNA was later purified and concentrated further using the Qiagen RNEasy Mini kit (Qiagen). Total RNA was submitted to the University of Wisconsin-Madison Gene Expression Center for cDNA synthesis, and Nimblegen conducted labeling and chip hybridization.

Sixty-mer oligo expression array chips were designed against all of the ORFs recognized in December 2007. Each oligo was present in five internal replicates. Expression array data were analyzed using \textsc{Arraystar} 2.0.

**Regulation of pentose phosphate pathway (PPP), glycolysis and tricarboxylic acid (TCA) cycle**

In \textit{P. stipitis} and other Crabtree-negative yeasts, fermentation is induced in response to oxygen limitation rather than the presence of glucose (Passoth et al., 1996; Kiers et al., 1998; Cho & Jeffries, 1999). Previous published studies on fermentation regulation in Crabtree-negative yeasts have apparently not included global transcriptional expression...
arrays. Describing the complete results would require a book rather than a short review, but Fig. 1 summarizes the expression levels of 88 transcripts of genes thought to be involved directly or indirectly with the PPP, glycolysis and TCA cycle, with cells grown on glucose or xylose under aerobic or oxygen-limited conditions. Note that the vertical axes of these graphs vary with each group of genes in order to show the relative changes of low-abundance transcripts.

Notably, about half of the transcripts do not appear to change significantly under these four cultivation conditions.

**Fig. 1.** Transcript expression levels of glycolytic enzymes in *Pichia stipitis*. Triplicate cultures were grown at 30 °C, pH 5, in 2 L of minimal defined medium with 50 g·L⁻¹ glucose (G) or xylose (X) under fully aerobic (A) or oxygen-limited (OL) conditions. Cell samples were harvested for mRNA, converted to cDNA and hybridized against 60-mer NimbleGen expression arrays. The average relative expression levels of normalized triplicate samples are shown. Gene designations follow the annotation posted at http://genome.jgi-psf.org/Picst3/Picst3.home.html
corresponding functions under these conditions are not fully understood, in part because their patterns. The three genes of the xylose assimilation pathway, XYL1, XYL2 and XYL3, are clearly upregulated on xylose under aerobic or oxygen-limited conditions, and are repressed or downregulated on glucose. Glucose repression of these three genes has been recognized for some time. What is interesting in the context of a global expression analysis is that at its peak, the expression of XYL2 is among the most abundant of all transcripts in the cell. Data suggest that other transcripts of the PPP are slightly elevated when cells are cultivated on xylose as opposed to glucose. While the changes might seem small, these activities are required under all growth conditions, and the transcript levels are high in all samples; hence, a 20% increase in transcript on xylose under aerobic or oxygen-limiting conditions is significant. Also, the effect is more convincing when similar changes are observed in more than half of the genes simultaneously. By a similar argument, transcripts for the glycolytic enzymes, Pfk1, Pfk2, Fba1, Tpi1, Pgk1, Gpm1.1 and Gpm1.2 all seem to show small increases on glucose, and to a lesser extent, xylose, under fermentative conditions.

Among the most dramatically regulated transcripts are those for triosephosphate dehydrogenase (TDH1, 2, 3 and 4). Whereas TDH3 is constitutive and among the most abundant of all transcripts, TDH2 is strongly induced on either xylose or glucose, and TDH1 is induced on glucose under oxygen limitation. This pattern of one isoform being expressed constitutively with other isoforms for the same activity induced under other conditions – presumably in response to increased metabolic demand – is reflected in PDC1 and PDC2, UGA1.1 and UGA1.2, MSL1.1 and MSL1.2 as well as other examples not described in this review. This suggests that something limits how much transcript can be produced by a single promoter on a single gene, and that to accommodate the higher levels required for growth, multiple copies evolve with different expression or kinetic patterns.

The abundance of transcripts for ADH1 and ADH2 are low relative to the levels of those for other glycolytic or fermentative enzymes and transcripts for the corresponding enzymes found in S. cerevisiae. These two activities are essential for ethanol production in P. stipitis because deletion of either one or the other significantly reduces ethanol formation, and deletion of both eliminates it almost entirely (Cho & Jeffries, 1998). The induction patterns for ADH4, ADH7 and SAD2 on glucose, xylose and under oxygen limitation are not fully understood, in part because their corresponding functions under these conditions are not known.

Regulatory pathways

Very little is as yet known about regulatory pathways in P. stipitis, and what has been gathered from expression array studies requires further confirmation by targeted deletions and physiological studies. With these caveats, however, we can project some possible pathways based on structural similarities and comparative functions in S. cerevisiae and other yeasts. Some regulatory proteins are fairly well conserved among P. stipitis, S. cerevisiae, Candida albicans and other fungi. The more conserved regulatory proteins such as Gcn1, Gcn2 and Gcn4 show strong one-to-one correlations with corresponding proteins in S. cerevisiae (Table 1), and their functions are assumed to be similar. Other proteins – some of which have well-studied functions in S. cerevisiae, such as Adr1, Hap1, Mig1 and Gcr1/Gcr2 – have only low (or no) similarity to proteins in P. stipitis, or they are similar to multiple proteins so a one-to-one correlation cannot be established. The genes in Table 1 are arranged in a rough descending order based on the degree of conservation observed between P. stipitis and S. cerevisiae. A comparison between P. stipitis and another more closely related yeast such as C. albicans would almost certainly show a higher degree of conservation, but because regulation has been best studied in S. cerevisiae, that is a standard frame of reference.

Genes for several proteins particularly play important idiomatic roles in S. cerevisiae, but they have no recognizable counterparts in P. stipitis. These include the Ino2 transcriptional activator of phospholipids, which could be involved in ethanol tolerance; Rgt1, which is involved in activating glucose transport; and Gcr1/Gcr2, which are transcriptional activators of glycolytic genes. Ethanol tolerance and high glycolytic flux are traits that have evolved as part of S. cerevisiae’s fermentative mode of growth and competitive survival in high-sugar environments. Because S. cerevisiae represses respiration in the presence of glucose whereas P. stipitis induces fermentative activities in response to oxygen limitation, differences in the glucose and oxygen regulatory pathways would be expected. Pichia stipitis has evolved in the relatively low-sugar environment prevailing in the beetle mid-gut; hence, its capacity for ethanol tolerance and high glycolytic flux are notably less.

Much of the S. cerevisiae pathway for sensing glucose seems to be conserved in P. stipitis. Relevant proteins showing high structural similarity include Rgt2, Grr1, Snf1, Sks1, Snf3, Mig2, Mig2.2 and Nrg1. Genes showing little or no conservation include ScMig1/CreA and ScAdr1. Proteins involved in the pathway for oxygen regulation are also conserved in P. stipitis. Proteins showing significant similarity include Hap5, Hap3 and Hap2. Proteins with weak or no significant identity in P. stipitis include ScHap1 and ScHap4.
Table 1. Regulatory genes in Saccharomyces cerevisiae with corresponding proteins in Pichia stipitis

| S. cerevisiae | P. stipitis | Description of protein function | Comments |
|--------------|-------------|---------------------------------|----------|
| GCN1         | GCN1        | Translational activator of GCN4  | Conserved protein |
| HAP5         | HAP5        | CCAAT-binding transcription factor | Conserved protein |
| DAL80        | GZF3        | Negative regulator of genes in multiple nitrogen degradation pathways | Conserved protein |
| SNF1         | SNF1        | AMP-activated serine/threonine protein kinase; required to transcribe glucose-repressed genes | Conserved in P. stipitis; 17 similar proteins |
| GRR1         | GRR1        | F-box protein component; involved in carbon catabolite repression, glucose-dependent divalent cation transport, high-affinity glucose transport, morphogenesis and sulfate detoxification | Well conserved in P. stipitis; one other gene with slight similarity |
| YAP1         | YAP1/CAP1   | Response to oxidative stress/oxygen detoxification | Moderately conserved |
| REB1         | REB1        | Involved in pol II and pol I transcription, repressor | Moderately conserved |
| HAP2         | HAP2        | Transcriptional activator | Moderately conserved |
| GCN4         | GCN4        | Transcriptional activator of amino acid biosynthesis | Relatively low identity |
| HAP4         | HAP4        | Positive regulator of cytochrome c genes | Multiple similar genes |
| MIG1         | MIG1        | Sequence-specific DNA-binding protein involved in glucose repression, regulated by SNF1 kinase and the GLC7 phosphatase | MIG1p has weak identity with nine proteins in P. stipitis |
| SWI14        | SWI14       | Mediates cell-cycle-dependent transcription of HO | Some similarity |
| SWI16        | SWI16       | Together with Swi4p, it forms the factor SBF | Similarity |
| XB1          | XB1         | Stress-induced repressor | Weak similarity |
| NRG1         | NRG1        | Transcriptional repressor for glucose repression | Similar to ScNRG1 |
| MSM4         | MSM4        | Regulates carbon source utilization | Similar to several |
| HAP1         | HAP1.1, 1.2, 1.3 | Fungal transcriptional regulatory protein, similar to CYP1/ HAP1 transcriptional activator | Multiple genes |
| HAP3         | HAP3.1, 3.2 | CCAAT-binding factor, subunit A | Multiple genes |
| GAL4         | GAL4/C13, LAC9 | DNA-binding transcription factor required for the activation of the GAL genes | Similarity to PsLAC9p and PsGAL4p |
| MIG2         | MIG2, MIG2.2 | Transcription factor involved in glucose repression | PsMIG2.2, immediately adjacent to PsGCN1 |
| ADR1         | ADR1.1, ZMS1 | Carbon source-responsive Zn-finger protein | Ten proteins show low similarity to ScADR1p |
| GLN3         | GLN3        | Transcriptional activator of genes regulated by nitrogen catabolite repression | Four similar proteins in P. stipitis |
| INO2, INO4   | INO4        | Transcription activator required for derepression of phospholipid biosynthetic genes | Slight similarity in P. stipitis |
| RGT1         | –           | Glucose-responsive transcription factor regulating expression of glucose transporters (HTT) | One protein with slight similarity in P. stipitis |
| GCR1, GCR2   | –           | Interactive DNA-binding heterodimer transcriptional activators of glycolytic genes | No corresponding proteins in P. stipitis |

The P. stipitis genome contains > 200 putative Zn-finger regulatory proteins, many of which are poorly conserved or annotated. Because 5’ sequences and regulatory processes appear to evolve much more rapidly than ORFs coding for enzymatic activities, the divergence and diversity of proteins for regulatory responses should not be surprising.

The regulatory pathway that induces fermentative enzymes in P. stipitis and other Crabtree-negative yeasts under oxygen-limiting conditions still needs to be resolved. In this regard, transcript levels for SKS1 and SNF3 are upregulated about twofold under oxygen-limited conditions on either glucose or xylose. Levels of GRR1 are essentially unchanged. Specific regulatory responses to glucose and xylose under oxygen limitation are complex, currently under analysis and will be the subject of future publications.

Functional gene clusters

Functional gene clusters are defined as physically proximal genes sharing common physiological or metabolic attributes. These fall loosely into two groups: (1) pairs or clusters of nonhomologous genes in which each cluster has a single function such as galactose metabolism, and (2) tandem repeats of paralogous genes. Functional clusters are not to be confused with structurally related genes across a range of species that constitute orthologous clusters (Dehal & Boore, 2006). Nor are they to be confused with operons that are prevalent in bacteria, because each gene possesses its own promoter and terminator. Rather, they represent a set of genes coding for proteins with physiologically related functions that confer a survival advantage when coinherited.
The most conspicuous functional clusters in *P. stipitis* consist of two or more families of homologs associated in different ways in different clusters. For the purpose of this review, we define gene families as proteins belonging to orthologous groups as evidenced by common InterPro domain architectures or consistent phylogenetic relationships. Proteins duplicating and diverging within a species are termed paralogs, while proteins having common functions in different species are termed orthologs. In *P. stipitis*, we have evidence that pairs or clusters of proteins having different orthologous origins have duplicated as clusters and diverged into different functions.

In some cases, genes in a functional cluster are coregulated. They often appear in subtelomeric regions where recombination occurs frequently. While gene clusters have been recognized in *S. cerevisiae* and other yeasts, they seem to be particularly abundant in *P. stipitis*. Cross-genomic comparisons show that some clusters for basic functions such as urea metabolism (Table 2) are widely conserved across species, while others for more idiomatic functions seem to be unique. Histones, for example, are present in four heterodimer pairs. These are not tandem duplications of individual genes. Rather, each pair consists of an H3-H4 or H2A-H2B subunit, presumably because they function in that manner. This appears to be a highly conserved cross-species cluster. Because clusters are so prominent in *P. stipitis*, a few hypotheses can be drawn from their features.

Sequencing of the *P. stipitis* genome (Jeffries et al., 2007) revealed the presence of at least 35 clusters of functionally related genes. Five occur in the subtelomeric regions of chromosomes (< 30 kbp from the end). At least 18 clusters consist of two or more genes having different enzymatic activities that are functionally related. For example, maltose permeases are found in association with α-glucosidases;
β-glucosidases are found in association with endoglucanases, and several genes for rhamnose metabolism, galactose metabolism, N-acetyl glucosamine degradation or pyrimidine metabolism are found adjacent to one another. In some instances, it is apparent that the pair or cluster formed, and then duplicated. In the case of the maltose permease/α-glucosidase pair, duplication of the gene pair was preserved.

Six other clusters consist of direct tandem repeats of divergent genes in ways similar to that reported in S. cerevisiae (Feuermann et al., 1997). The gene clusters listed in Table 2 have been found by inspecting the genome; many more examples should be apparent from more exacting bioinformatic approaches.

In many cases, the clusters of genes having different activities but related functions are each themselves members of divergent orthologous families. For example BGL, EGC, SUT, HXT and maltose (MAL) (Jeffries, 2008) gene families are associated with one another in various combinations. By constructing dendrograms of each of these gene families, it is possible to discern that in some instances (e.g. α-glucosidase/maltose permease and β-glucosidases/hexose transporter), the clusters appear to have formed and then the pairs of genes diverged together. In other instances, divergent members of these gene families are found unassociated with functionally related proteins (e.g. BGL7, BGL4, EGC3 and HXT2.1).

As noted in Table 2, any one pair of genes can be present in convergent, divergent or tandem orientations with respect to the direction of their transcription. The divergent orientation, in which two genes with related functions share a common 5′ sequence, has some implication with respect to regulation. This is the case with the α-glucosidase/maltose permease pairs. The orientation (and spacing) of the two genes has been maintained as the gene pair has duplicated. A comparison of the phylogenies for these two gene families gave rise to a series of diverging genes: YIC1/MAL5, ⇒ MAL9/MALx ⇒ AGL1/MAL3 ⇒ MAL8/MAL4 ⇒ MAL7/MAL2 ⇒ MAL6/MAL1. In the process, MAL9 appears to have lost its associated permease and is now associated with a completely different transport protein (Fig. 2).

The regulatory profiles of gene clusters exhibit patterns that are characteristic of the genes with which they are associated and their evolutionary divergence. For example, on at least one occasion, a complete triplet cluster of β-glucosidases duplicated (cf. BGL1/HXT2.6 and BGL3/HXT2.5 clusters, Fig. 3). In this case, the transcriptional profiles of the two clusters are almost identical, with the genes strongly induced in the presence of cellobiose. Moreover, phylogenetic comparisons of the Hxt, Sut and Bgl protein families show that these protein pairs have barely diverged from one another (Fig. 3). A phylogenetic comparison of the seven putative β-glucosidases in P. stipitis shows that BGL4 diverged earliest. This enzyme is most similar to fungal gentiobiases (β-1,6 glucosidase). It is expressed only at a very low level.

Phylogenetic comparisons of β-glucosidases associated with the endoglucanases show different regulatory profiles.
The EGC3/HXT2.1 pair might have diverged before its duplication and association with BGL5. The EGC2/BGL5 part of the triplicate cluster then appears to have duplicated once again to form the EGC1/BGL6 pair. The regulatory profile of BGL7 is quite different from those of BGL1 and BGL3, but quite similar to those of BGL5 and BGL2. In the triplet cluster on chromosome 1, the HXT2.4 and BGL5 expression profiles are very similar, but EGC2 is expressed to

Fig. 3. Correlation of gene clusters, phylogeny and expression for endo-glucanases, β-glucosidases and sugar transporters in Pichia stipitis. Each of the physical gene clusters are indicated by the colored blocks, and the phylogenetic relationships of the families are shown in the dendograms below. In addition to cultivation on glucose (G) or xylose (X) under aerobic (A) or oxygen-limited (OL) conditions, cells were cultivated in bioreactors on cellobiose (Cello) or L-arabinose (Ara) under aerobic conditions. Other features are as per Figs 1 and 2.
a lesser extent, suggesting that it might have been inserted between a progenitor of BGL2/HXT2.3. With the divergence of EGC1/BGL6, induced production of the endoglucanase in response to cellobiose and arabinose became much more pronounced, and the expression profile of BGL6 evolved in a similar manner.

In a number of clusters, and most conspicuously in those that appear to have evolved in longest association with one another, the genes have a divergent orientation. Transcription proceeds in either direction from a central shared promoter sequence. This is true in all of the MAL gene clusters, several of the BGL gene clusters, the dityrosine cluster, the histone clusters and the GAL1-GAL10 cluster. Many of these highly evolved clusters are found over a wide taxonomic distance.

Examples of gene clusters are not confined to P. stipitis. Indeed, some of the most conspicuous clusters such as DUR3-DUR1 (for urea metabolism), GAL1-GAL10-GAL7 and the MAL1 locus are found in S. cerevisiae, D. hansenii and other yeasts. Interestingly, the P. stipitis cluster contains a fourth gene, GAL102, which codes for a putative UDP-glucose 4-epimerase, along with a gene with unknown function (Table 2). Likewise, in comparison with the MAL11, MAL12, MAL13 locus of S. cerevisiae, which contains the regulatory GAL4-like Zn-finger protein, MAL13, along with a maltose permease (MAL11) and an α-glucosidase (MAL12), the corresponding P. stipitis locus (Fig. 2) has two putative permaeses and two highly divergent glucosidases – one of which (YIC1) is similar in structure to a xyllosidase. Two fungal-specific Zn-finger proteins belonging to the same family as GAL4 and MAL13 flank these four genes in P. stipitis. Likewise, the MAL2-MAL7 cluster has a flanking gene for a Zn-finger protein. The roles of these flanking Zn-finger genes and their associated proteins in regulation are unclear.

The dityrosine metabolic cluster, which codes for genes related to spor formation, is coregulated, as one might expect. These are coregulated with AUT4 for autophagy, which also happens to be colocated in the genome, along with a chitinase, which would function to hydrolyze the vegetative cell wall. These genes are downregulated on cellbiose aerobic or xylose under oxygen limitation. The NAG1, 2 and 4 genes likewise show similar regulation properties.

Table 2 is not exhaustive and consists principally of gene clusters that have been recognized in the course of annotating the genome. Indeed, Watanabe et al. (2008a, b) discovered a cluster of four genes for an alternative D-rhamnose metabolic pathway, even though several of the genes in the cluster did not have a known function before their study (Koivistoinen et al., 2008). Bioinformatic studies combining genomic and expression array data will probably reveal more unknown metabolic associations and pathways.

Clearly, much more can be learned about regulation of genes relevant to lignocellulose bioconversion through the comparative study of gene clusters in different yeasts. Gene clusters often appear to be coregulated with genes in each cluster showing similar induction patterns. Gene clusters such as MAL6 and MAL1, which appear to be highly specific for maltose utilization, are highly induced when cells are grown on maltose, while other clusters show broader patterns of induction. A more comprehensive comparison of MAL, GAL and DUR clusters across taxonomic lines could provide a better picture of how these traits have evolved in yeasts.

**Tandem duplication**

Tandem duplication appears to be an early stage in the formation of gene families. It is not entirely clear how new genes either enter the genome or become amplified. Some appear to be acquired through lateral transfer from other yeasts, other fungi or bacteria.

The OYE gene family, which codes for NADPH dehydrogenase, is present in 12 members, six of which appear in two triplet clusters of tandem repeats. OYE genes are present in other related genomes. Debaryomyces hansenii has an even larger cluster of OYE genes, five in all, that are found on Chromosome C (Dujon et al., 2004). Three of these are very similar to PsOYE2.5, 2.5 and 2.8 on PsCh 4. These clusters correspond to a similar OYE cluster in Pichia guilliermondii (PGUG_05760-PGUG_05763) (Broad Institute 2009), which is flanked by putative NAD/NADP-linked alcohol dehydrogenases proximal to either end (PGUG_05764 and PGUG_05758). A similar ADH (PsADH5) is proximal to the triplet OYE cluster in P. stipitis. Phylogenetic analysis of the P. stipitis OYE genes suggests that a triplicate cluster underwent three rounds of duplication, followed by differentiation with little or no loss of the intermediate activities. Regulatory profiles of the tandem genes show little similarity except among genes that have recently duplicated. The proliferation and differentiation of these genes probably represents the fulfillment of new metabolic niches. *Pichia stipitis* occurs in both free-living and insect-associated forms, and the different NADH dehydrogenases would be required for these adaptations.

Likewise, the AKR family of aldo/keto reductases – to which XYL1 for xylose reductase belongs – is present in six members with two paired clusters. Survival of duplicated pairs and triplicates in the genome implies that higher levels of these enzymatic activities were required for survival of the yeast in its habitat.

Cinnamyl alcohol reductases (CAD) are present in five copies, of which two (CAD2/CAD3) exist in a tandem repeat. CAD5 is the most divergent. Very few examples can be found in related genomes for this enzymatic activity. This, therefore, represents an idiomatic activity. Tandem duplication seems to be characteristic of newly acquired
genes for which expression levels are low. Following duplication, proteins stemming from a common function could evolve into enzymes with different kinetic properties that would improve cellular metabolism. Speculation about the functions of these multiple genes is not really warranted in the absence of additional biochemical, physiological study, but the emergence, proliferation and divergence of such clusters into gene families are certainly of interest.

The largest gene cluster of tandem repeats thus far recognized in *P. stipitis* is a set of five genes coding for apparent 2′ hydroxyisoflavone reductases (*CIP1.1-1.5*). Homologs with weak similarity are found in two copies in *D. hansenii* and *Yarrowia lipolytica*, but the cluster of *CIP1* genes appears to be idiomatic to *P. stipitis*. The function of this enzymatic activity in *P. stipitis* is unknown. Four out of five gene copies are transcribed minimally under all of the growth conditions tested. One copy, however, *CIP1.1*, is expressed at significant levels and has evolved through selection much more than the others. Because the five direct repeats are unusual in their repetitive nature, it is possible to trace the evolutionary divergence of the 5′ sequences in addition to the ORFs. As can be seen in Fig. 4, the evolutionary divergence of the promoter sequences parallel those of the structural genes, but at about twice the rate. The promoter of *CIP1.1* apparently acquired several 12–24-bp segments not found in the other sequences. These presumably increased the expression and allowed selection to work more effectively on this paralog.

**Resequencing – what we learned about selection**

Direct and complete resequencing of the *P. stipitis* genome after several rounds of mutagenesis, selection and cultivation over several years has provided an insight into how adaptation occurs at a molecular level. Surprisingly, only 14 point mutations were preserved during this process (Smith et al., 2008). Of these, 10 were in ORFs and four were in intergenic regions.

All of the mutations in ORFs resulted in amino acid substitutions, and seven of these resulted in significant changes in the amino acid function. The degeneracy of the genetic code often preserves the same amino acid residue when mutations occur in the third position, and the structure of the code tends to conserve hydrophobic, hydrophilic, acidic, basic and other amino acid characteristics even when substitutions occur; hence, the prevalence of mutations altering amino acid function has a very low probability of being attributable to random events.

---

**Fig. 4. CIP1 gene cluster.** (a) Genomic organization of the cluster. (b) Gene expression data. Triplicate cultures were grown at 30°C, pH 5, in 2 L of minimal defined medium with 50 g L^-1^ glucose (G), xylose (X), cellobiose (Cel) or arabinose (Ara) under fully aerobic (A) or oxygen-limited (OL) conditions. Cell samples were harvested for mRNA, converted to cDNA and hybridized against 60-mer NimbleGen expression arrays. The average relative expression levels of normalized triplicate samples are shown. (c) Different rates of evolutionary divergence for CIP1 genes in a tandemly duplicated cluster. Protein sequences and the 5′ sequences were separately aligned using CLUSTAL W and phylogenies were calculated using the best tree neighbor-joining and the Poisson correction.
Selection for resistance to inhibitors, growth on limiting carbon sources and selection for more rapid growth or fermentation was used at each stage in the selection process; hence, the resulting mutations probably provided adaptive advantages for growth under the selective conditions used. Unfortunately, our limited knowledge of the functions of the affected genes does not permit a rational interpretation of the genotypes.

**Conclusions**

Similar gene clusters and regulatory patterns could probably be observed in many different yeasts. Their recognition in *P. stipitis* is the result of concerted annotation and expression array studies. What we can see from this, however, is that gene families appear to arise from acquisition, duplication and differentiation within a genome. When complementary activities become colocated through the process of resortment, evolution of the cluster can proceed through duplication and further codifferentiation. *Cis*-acting regulatory features can evolve more rapidly and can be coordinated across several genes in a cluster. In this process, a divergent gene orientation with common 5’ sequences tends to be preserved. Concerted studies of structure, function and regulation in yeast genomes are likely to result in many more insights into cell physiology and evolution.

**Acknowledgement**

The authors wish to acknowledge funding for this project from the USDA, CSREES NRI project #2006-355-04-17436.

**Statement**

Reuse of this article is permitted in accordance with the Creative Commons Deed, Attribution 2.5, which does not permit commercial exploitation.

**References**

Alexander MA, Chapman TW & Jeffries TW (1987) Continuous ethanol production from D-xylose by *Candida shehatae*. Biotechnol Bioeng 30: 685–691.

Basaran P & Ozcan M (2008) Characterization of beta-xylosidase enzyme from a *Pichia stipitis* mutant. *Bioresource Technol* **99**: 38–43.

Basaran P, Hang YD, Basaran N & Worobo RW (2001) Cloning and heterologous expression of xylanase from *Pichia stipitis* in *Escherichia coli*. *J Appl Microbiol* **90**: 248–255.

Bicho PA, Runnals PL, Cunningham JD & Lee H (1988) Induction of xylose reductase and xylitol dehydrogenase activities in *Pachymanulum tannophilus* and *Pichia stipitis* on mixed sugars. *Appl Environ Microb* **54**: 50–54.

Cho JY & Jeffries TW (1998) *Pichia stipitis* genes for alcohol dehydrogenase with fermentative and respiratory functions. *Appl Environ Microb* **64**: 1350–1358.

Cho JY & Jeffries TW (1999) Transcriptional control of ADH genes in the xylose-fermenting yeast *Pichia stipitis*. *Appl Environ Microb* **65**: 2363–2368.

Conceicao GIA, Moran PJS & Rodrigues JAR (2003) Highly efficient extractive biocatalysis in the asymmetric reduction of an acyclic enone by the yeast *Pichia stipitis*. *Tetrahedron-Asymmetr* **14**: 43–45.

Dahn KM, Davis BP, Pittman PE, Kenearly WR & Jeffries TW (1996) Increased xylose reductase activity in the xylose-fermenting yeast *Pichia stipitis* by overexpression of XYL1. *Appl Biochem Biotechnol* **57–8**: 267–276.

Dehal PS & Boore JL (2006) A phylogenetic gene cluster resource: the Phylogenetically Inferred Groups (PhIGs) database. *BMC Bioinformatics* **7**: 201.

Dujon B, Sherman D, Fischer G et al. (2004) Genome evolution in yeasts. *Nature* **430**: 35–44.

du Preez JC, van Driessel B & Prior BA (1989) Ethanol tolerance of *Pichia stipitis* and *Candida shehatae* strains in fed-batch cultures at controlled low dissolved-oxygen levels. *Appl Microbiol Biot* **30**: 53–58.

Feurmann M, DeMontigny J, Potier S & Souciet JL (1997) The characterization of two new clusters of duplicated genes suggests a ‘Lego’ organization of the yeast *Saccharomyces cerevisiae* chromosomes. *Yeast* **13**: 861–869.

Fuganti C, Grasselli P, Zucchi G, Allegrone G & Barbeni M (1993) On the microbial biogeneration of (R) gamma-jasmonolactone. *Bioorg Med Chem Lett* **3**: 2777–2780.

Gorgens JF, Passoth V, van Zyl WH, Knoetze JH & Hahn-Hagerdal B (2005) Amino acid supplementation, controlled oxygen limitation and sequential double induction improves heterologous xylanase production by *Pichia stipitis*. *FEMS Yeast Res* **5**: 677–683.

Guptar AS (1994) Theoretical and practical aspects of ploidy estimation in *Pichia stipitis*. *Mycol Res* **98**: 716–718.

Hahn-Hagerdal B & Pamment N (2004) Microbial pentose metabolism. *Appl Biochem Biotechnol* **113–16**: 1207–1209.

Harhangi HR, Akhmanova AS, Emmens R et al. (2003) Xylose metabolism in the anaerobic fungus *Piromyces* sp strain E2 follows the bacterial pathway. *Arch Microbiol* **180**: 134–141.

Hector RE, Qureshi N, Hughes SR & Cotta MA (2008) Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. *Appl Microbiol Biot* **80**: 675–684.

Ho NWY, Chen ZD & Brainard AP (1998) Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl Environ Microb* **64**: 1852–1859.

**Broad Institute** (2009) *Genome Description for Pichia guilliermondii*. Broad Institute, Cambridge, MA. Available from http://www.broad.mit.edu/annotation/genome/candida_group/GenomeDescriptions.html#C_guilliermondii

**Harhangi HR, Akhmanova AS, Emmens R et al. (2003)** Xylose metabolism in the anaerobic fungus *Piromyces* sp strain E2 follows the bacterial pathway. *Arch Microbiol* 180: 134–141.

**Hector RE, Qureshi N, Hughes SR & Cotta MA (2008)** Expression of a heterologous xylose transporter in a *Saccharomyces cerevisiae* strain engineered to utilize xylose improves aerobic xylose consumption. *Appl Microbiol Biot* 80: 675–684.

**Ho NWY, Chen ZD & Brainard AP (1998)** Genetically engineered *Saccharomyces* yeast capable of effective cofermentation of glucose and xylose. *Appl Environ Microb* 64: 1852–1859.
Ilmen M, Koivuranta K, Ruohonen L, Suominen P & Penttila M (2007) Efficient production of \( \alpha \)-lactic acid from xylose by *Pichia stipitis*. *Appl Environ Microb* 73: 117–123.

Jeffries TW (2008) Engineering the *Pichia stipitis* genome for fermentation of hemicellulose hydrolysates. *Bioenergy* (Wall JD, Harwood CS & Demain A, eds), pp. 37–47. ASM Press, Washington, DC.

Jeffries TW & Jin YS (2004) Metabolic engineering for improved fermentation of pentoses by yeasts. *Appl Microbiol Biot* 63: 495–509.

Jeffries TW, Grigoriev IV, Grimwood J et al. (2007) Genome sequence of the lignocellulose-bioconverting and xylose-fermenting yeast *Pichia stipitis*. *Nat Biotechnol* 25: 319–326.

Jin YS, Jones S, Shi NQ & Jeffries TW (2002) Molecular cloning of XYL3 (\( \beta \)-xyulokinase) from *Pichia stipitis* and characterization of its physiological function. *Appl Environ Microb* 68: 1232–1239.

Jin YS, Laplaza JM & Jeffries TW (2004) *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. *Appl Environ Microb* 70: 6816–6825.

Kahrumaa K, Hahn-Hagerdal B & Gorwa-Grauslund MF (2005) Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. *Yeast* 22: 359–368.

Kahrumaa K, Sanchez RG, Hahn-Hagerdal B & Gorwa-Grauslund MF (2007) Comparison of the xylose reductase-xyitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Microb Cell Fact* 6.

Kawahira S, Ito M, Takema H et al. (2008) Improvement of ethanol productivity during xylose and glucose co-fermentation by xylose-assimilating *S. cerevisiae* via expression of glucose transporter Sut1. *Enzyme Microb Tech* 43: 115–119.

Kiers J, Zeeman AM, Luttik M et al. (1998) Regulation of alcoholic fermentation in batch and chemostat cultures of *Kluyveromyces lactis* CBS 2359. *Yeast* 14: 459–469.

Kim MS, Chung YS, Seo JH, Jo DH, Park YH & Ryu YW (2001) High-yield production of xyitol from xylose by a xyitol dehydrogenase defective mutant of *Pichia stipitis*. *J Microbiol Biotechn* 11: 564–569.

Klabunde J, Kunze G, Gelissen G & Hollenberg CP (2003) Integration of heterologous genes in several yeast species using vectors containing a *Hansenula polymorpha*-derived rDNA-targeting element. *FEMS Yeast Res* 4: 185–193.

Kliver U, Fluthgraf S, Freese S & Passoth V (2005) Aerobic induction of respiro-fermentative growth by decreasing oxygen tensions in the respiratory yeast *Pichia stipitis*. *Appl Microbiol Biot* 67: 247–253.

Koivistoinen OM, Hilditch S, Voutilainen SP, Boer H, Penttila M & Richard P (2008) Identification in the yeast *Pichia stipitis* of the first \( \alpha \)-rhamnose-1-dehydrogenase gene. *FEBS J* 275: 2482–2488.

Kotter P, Amore R, Hollenberg CP & Ciriaci M (1990) Isolation and characterization of the *Pichia stipitis* xyitol dehydrogenase gene, XYL2, and construction of a xylose-utilizing *Saccharomyces cerevisiae* transformant. *Curr Genet* 18: 493–500.

Kurtzman CP (1990) *Candida shehatae* — genetic diversity and phylogenetic relationships with other xylose-fermenting yeasts. *Antonie van Leeuwenhoek* 57: 215–222.

Laplaza JM, Torres BR, Jin YS & Jeffries TW (2006) Sh ble and Cre adapted for functional genomics and metabolic engineering of *Pichia stipitis*. *Enzyme Microb Tech* 38: 741–747.

Leandro MJ, Spencer-Martins I & Goncalves P (2008) The expression in *Saccharomyces cerevisiae* of a glucose/xylose symporter from *Candida intermedia* is affected by the presence of a glucose/xylose facilitator. *Microbiology-Sgm* 154: 1646–1655.

Lee H, Biely P, Latta RK, Barbosa MFS & Schneider H (1986) Utilization of xylan by yeasts and its conversion to ethanol by *Pichia stipitis* strains. *Appl Environ Microb* 52: 320–324.

Liu ZL, Slininger PJ & Gorsich SW (2005) Enhanced biotransformation of furfural and hydroxymethylfurfural by newly developed ethanologenic yeast strains. *Appl Microbiol Biot* 121: 451–460.

Lu P, Davis BP, Hendrick J & Jeffries TW (1998a) Cloning and disruption of the beta-isopropylmalate dehydrogenase gene (LEU2) of *Pichia stipitis* with URA3 and recovery of the double auxotroph. *Appl Microbiol Biot* 49: 141–146.

Lu P, Davis BP & Jeffries TW (1998b) Cloning and characterization of two pyruvate decarboxylase: genes from *Pichia stipitis* CBS 6054. *Appl Environ Microb* 64: 94–97.

Maasen N, Freese S, Schruff B, Passoth V & Kliner U (2008) Nonhomologous end joining and homologous recombination DNA repair pathways in integration mutagenesis in the xylose-fermenting yeast *Pichia stipitis*. *FEBS Yeast Res* 8: 735–743.

Manzanares P, Ramon D & Querol A (1999) Screening of non-*Saccharomyces* wine yeasts for the production of beta-\( \text{D} \)-xylosidase activity. *Int J Food Microb* 46: 105–112.

Matsushika A, Watanabe S, Kodaki T, Makino K & Sawayama S (2008) Bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* expressing xylose reductase, NADP(+)-dependent xyitol dehydrogenase, and xyulokinase. *J Biosci Bioeng* 105: 296–299.

Melake T, Passoth V & Kliner U (1996) Characterization of the genetic system of the xylose-fermenting yeast *Pichia stipitis*. *Curr Microbiol* 33: 237–242.

Nardi JB, Bee CM, Miller LA, Nguyen NH, Suh SO & Blackwell M (2006) Communities of microbes that inhabit the changing hindgut landscape of a subsocial beetle. *Arthropod Struct Dev* 35: 57–68.

Nigam JN (2001a) Development of xylose-fermenting yeast *Pichia stipitis* for ethanol production through adaptation on hardwood hemicellulose acid prehydrolysate. *J Appl Microbiol* 90: 208–215.

Nigam JN (2001b) Ethanol production from hardwood spent sulfite liquor using an adapted strain of *Pichia stipitis*. *J Ind Microbiol Biot* 26: 145–150.

Ozcan S, Kotter P & Ciriaci M (1991) Xylan-hydrolyzing enzymes of the yeast *Pichia stipitis*. *Appl Microbiol Biot* 36: 190–195.
Passoth V & Hahn-Hagerdal B (2000) Production of a heterologous endo-1,4-beta-xylanase in the yeast Pichia stipitis with an O-2-regulated promoter. Enzyme Microb Tech 26: 781–784.

Passoth V, Hansen M, Klinner U & Emeis CC (1992) The electrophotoretic banding patterns of the chromosome of Pichia stipitis and Candida shehatae. Curr Genet 22: 429–431.

Passoth V, Zimmermann M & Klinner U (1996) Peculiarities of the regulation of fermentation and respiration in the crabtree-negative, xylose-fermenting yeast Pichia stipitis. Appl Biochem Biotechnol 57–8: 201–212.

Passoth V, Schafer B, Liebel B, Weierstall T & Klinner U (1998) Molecular cloning of alcohol dehydrogenase genes of the yeast Pichia stipitis and identification of the fermentative ADH. Yeast 14: 1311–1325.

Passoth V, Cohn M, Schafer B, Hahn-Hagerdal B & Klinner U (2000) Molecular analysis of the hypoxia induced ADH2 promoter in the respiratory yeast Pichia stipitis. Yeast 20: S199–S199.

Passoth V, Cohn M, Schafer B, Hahn-Hagerdal B & Klinner U (2003a) Analysis of the hypoxia-induced ADH2 promoter of the respiratory yeast Pichia stipitis reveals a new mechanism for sensing of oxygen limitation in yeast. Yeast 20: 39–51.

Petschacher B & Nidetzky B (2008) Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of Saccharomyces cerevisiae. Microb Cell Fact 7.

Piontek M, Hagedorn J, Hollenberg CP, Gellissen G & Strasser AW (1998) Two novel gene expression systems based on the yeasts Schwanniomyces occidentalis and Pichia stipitis. Appl Microbiol Biot 50: 331–338.

Richard P, Tovari MH & Penttila M (2000) The role of xyulokinase in Saccharomyces cerevisiae xylulose catabolism. FEMS Microbiol Lett 190: 39–43.

Rodrigues R, Lu CF, Lin B & Jeffries TW (2008) Fermentation kinetics for xyitol production by a Pichia stipitis d-Xyulokinase mutant previously grown in spent sulfite liquor. Appl Biochem Biotechnol 148: 199–209.

Saloseimo A, Rauta J, Stasyk OV, Sibirny AA, Penttila M & Ruohonен L (2007) Xylose transport studies with xylose-utilizing Saccharomyces cerevisiae strains expressing heterologous and homologous permeases. Appl Microbiol Biot 74: 1041–1052.

Shi NQ, Davis B, Sherman F, Cruz J & Jeffries TW (1999) Disruption of the cytochrome c gene in xylose-utilizing yeast Pichia stipitis leads to higher ethanol production. Yeast 15: 1021–1030.

Shi NQ, Prahl K, Hendrick J et al. (2000) Characterization and complementation of a Pichia stipitis mutant unable to grow on D-xylose or D-arabinose. Appl Biochem Biotechnol 84–6: 201–216.

Shi NQ, Cruz J, Sherman F & Jeffries TW (2002) SHAM-sensitive alternative respiration in the xylose-metabolizing yeast Pichia stipitis. Yeast 19: 1203–1220.

Smith DR, Quinlan AR, Peckham HE et al. (2008) Rapid whole-genome mutational profiling using next-generation sequencing technologies. Genome Res 18: 1638–1642.

Sonderegger M, Jeppsson M, Hahn-Hagerdal B & Sauer U (2004) Molecular basis for anaerobic growth of Saccharomyces cerevisiae on xylose, investigated by global gene expression and metabolic flux analysis. Appl Environ Microbiol 70: 2307–2317.

Sugita T & Nakase T (1999) Non-universal usage of the leucine CUG codon and the molecular phylogeny of the genus Candida. Syst Appl Microbiol 22: 79–86.

Su H, Marshall CJ, McHugh JV & Blackwell M (2003) Wood ingestion by passalid beetles in the presence of xylose-fermenting gut yeasts. Mol Ecol 12: 3137–3145.

Takuma S, Nakashima N, Tantirungkij M, Kinoshita S, Okada H, Seki T & Yoshida T (1991) Isolation of xylose reductase gene of Pichia stipitis and its expression in Saccharomyces cerevisiae. Appl Biochem Biotechnol 28–9: 327–340.

Targonski Z (1992) Biotransformation of lignin-related aromatic-compounds by Pichia stipitis Pignal. Zbl Mikrobiol 147: 234–249.

van Dijken JP, van den Bosch E, Hermans JJ, de Miranda LR & Scheffers WA (1986) Alcoholic fermentation by ‘non-fermentative’ yeasts. Yeast 2: 123–127.

van Maris AJA, Winkler AA, Kuypers M, de Laat W, van Dijken JP & Pronk JT (2007) Development of efficient xylose fermentation in Saccharomyces cerevisiae: Xylose isomerase as a key component. Adv Biochem Eng Biotechnol, Vol. 108, pp. 179–204. Springer.

Vaughan Martini AE (1984) Comparazione dei genomi del lievito Pichia stipitis e de alcune specie imperfette affini. Ann Fac Agr Univ Perugia 38B: 331–335.

Verduyn C, Postma E & Scheffers WA (1992) Effect of benzoic acid on metabolic fluxes in yeasts - a continuous culture study on the regulation of respiration and alcoholic fermentation. Yeast 8: 501–517.

Watanabe S, Piyanart S & Makino K (2008a) Eukaryotic and bacterial gene clusters related to an alternative pathway of L-rhamnose metabolism. Yeast 25: 275–284.

Watanabe S, Piyanart S & Makino K (2008b) Eukaryotic and bacterial gene clusters related to an alternative pathway of L-rhamnose metabolism. Zbl Mikrobiol 25: 327–340.

Watanabe S, Saimura M & Makino K (2008a) Metabolic fate of l-lactaldehyde derived from an alternative L-rhamnose pathway. FEBS J 275: 5139–5149.

Watanabe S, Saimura M & Makino K (2008b) Eukaryotic and bacterial gene clusters related to an alternative pathway of nonphosphorylated L-rhamnose metabolism. J Biol Chem 283: 20372–20382.

Weierstall T, Hollenberg CP & Boles E (1999) Cloning and characterization of three genes (SUT1-3) encoding glucose transporters of the yeast Pichia stipitis. Mol Microbiol 31: 871–883.

Wiedemann B & Boles E (2008) Codon-optimized bacterial genes improve L-arabinose fermentation in recombinant Saccharomyces cerevisiae. Appl Environ Microbiol 74: 2043–2050.

Wiedemann B, Boles E & Keller M (2006) Construction and optimization of pentose-fermenting yeast strains for bioethanol production. Zuckerindustrie 131: 627–631.

Yang VW, Marks JA, Davis BP & Jeffries TW (1994) High efficiency transformation of Pichia stipitis based on its URA3 gene and a homologous autonomous replication sequence, ARS2. Appl Environ Microbiol 60: 4245–4254.