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Characterization of the xylose-transporting properties of yeast hexose transporters and their influence on xylose utilization

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For an economically feasible production of ethanol from plant biomass by microbial cells, the fermentation of xylose is important. As xylose uptake might be a limiting step for xylose fermentation by recombinant xylose-utilizing *Saccharomyces cerevisiae* cells a study of xylose uptake was performed. After deletion of all of the 18 hexose-transporter genes, the ability of the cells to take up and to grow on xylose was lost. Reintroduction of individual hexose-transporter genes in this strain revealed that at intermediate xylose concentrations the yeast high- and intermediate-affinity transporters Hxt4, Hxt5, Hxt7 and Gal2 are important xylose-transporting proteins. Several heterologous monosaccharide transporters from bacteria and plant cells did not confer sufficient uptake activity to restore growth on xylose. Overexpression of the xylose-transporting proteins in a xylose-utilizing PUA yeast strain did not result in faster growth on xylose under aerobic conditions nor did it enhance the xylose fermentation rate under anaerobic conditions. The results of this study suggest that xylose uptake does not determine the xylose flux under the conditions and in the yeast strains investigated.

Keywords: *Saccharomyces cerevisiae*, xylose uptake, glucose uptake, xylose fermentation, heterologous expression

INTRODUCTION

In common fermentation processes, the yeast *Saccharomyces cerevisiae* is widely used for biotechnological ethanol production. However, *S. cerevisiae* is only able to ferment hexose sugars, and not pentose sugars like xylose. Along with glucose, xylose is one of the major monosaccharide components of lignocellolosic biomass (Hayn *et al.*, 1993). For an economically feasible industrial process for ethanol production from plant biomass in lignocellulose hydrolysates, it is necessary to ferment all sugars present (von Sivers & Zacchi, 1995).

Efforts to establish a xylose-utilizing pathway in *S. cerevisiae* by insertion of the genes encoding xylose reductase and xylitol dehydrogenase from *Pichia stipitis* or other organisms have resulted in only poor ethanol production from xylose (Kötter & Ciriacy, 1993; Tantirungkij *et al.*, 1993; Walfridsson *et al.*, 1995). Various steps, including the uptake of xylose, have been suggested to limit the metabolism of xylose in metabolically engineered *S. cerevisiae* (Kötter & Ciriacy, 1993; Eliasson *et al.*, 2000). Uptake of xylose by *S. cerevisiae* has been proposed to be mediated more or less unspecifically by its hexose-transport system. This is composed of a large family of 18 related transporter proteins called Hxts and additional sugar transporters with broader substrate specificity (Boles & Hollenberg, 1997; Wieczorke *et al.*, 1999). In xylose-uptake experiments with *S. cerevisiae* cells, two kinetically distinct uptake components with K_m values of about 0·19 M and 1·5 M were determined, demonstrating that the monosaccharide transport system in *S. cerevisiae* has nearly a 200-fold lower affinity for xylose than for glucose (Kotyk, 1967; Lagunas *et al.*, 1982; Kötter & Ciriacy, 1993).

To improve the xylose-uptake activity of *S. cerevisiae* it is necessary to identify the specific transporters mediating uptake of xylose. Additionally, heterologous xylose transporters from other organisms may be expressed in *S. cerevisiae* to improve xylose uptake. Using *S. cerevisiae* TMB3201, expressing a functional xylose utilization pathway but lacking the complete monosaccharide-transport system, we have characterized the xylose-transporting properties of monosaccharide transporters

from yeast, bacteria and plants, by growth and [^{14}C]xylose uptake. Especially the high-affinity glucose transporters of *S. cerevisiae* can mediate uptake of xylose. Nevertheless, overexpression of these transporters in the xylose-fermenting yeast strain PUA-X1,2 did not increase its growth rate on xylose nor its xylose fermentation rate.

METHODS

Yeast strains and growth conditions. Yeast strains were: CEN.PK2-1C (*MAT***a** *leu2-3,112 ura3-52 trp1-289 his3-Δ*1 *MAL2-8*^{c} *SUC2*), EBY.VW4000 (*MAT***a** *Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8*^{c} *SUC2*) (Wieczorke *et al*., 1999), TMB3001 (*MAT***a** *his3-Δ*1::*YIpXR/XDH/XK MAL2-8*^{c} *SUC2*) (Eliasson *et al*., 2000), TMB3201 (*MAT***a** *Δhxt1-17 Δgal2 Δstl1 Δagt1 Δmph2 Δmph3 leu2-3,112 ura3-52 trp1-289 his3-Δ1*::*YIpXR/XDH/XK MAL2-8*^{c} *SUC2*) (this work), PUA-X1,2 [*his3-11 ura3-52 leu2*::*(LEU2:PDC1pr-XYL1, ADH1pr-XYL2)*] (kindly provided by T. Weierstall, Düsseldorf). Synthetic media consisted of 6·7 g l^{-1} Difco yeast nitrogen base (YNB) supplemented with amino acids and adenine, with or without uracil as a plasmid-selection marker, and with various carbon sources. Yeast cells were grown aerobically at 30 °C on a rotary shaker or on agar plates. Xylose fermentation was investigated by incubating 50 ml yeast cultures with a high cell density in sealed 50 ml bottles under gentle stirring.

Construction of plasmids. The 0·4 kb *Sac*I/*Spe*I *MET25* promoter fragment of multicopy plasmid p426MET25 (Mumberg *et al*., 1994) was replaced by a 0·4 kb DNA fragment containing an *HXT7* promoter fragment from −392 bp to −1 bp that was amplified by PCR with primers P426H7-1 (5′-CTAGAGCTCG TAGGAACAAT TTCGG-3′) and P426H7-2 (5′-CGACTAGTGT GATGGTGATG GTGATGCATG TTAACTTTTT GATTAAAATT AAAAAAACTT-3′), and plasmid YEpkHXT7 (Krampe *et al*., 1998) as the template, resulting in plasmid p4H7. All monosaccharide-transporter genes were cloned by recombination-cloning into p4H7 using the strategies described by Wieczorke *et al*. (1999). The yeast hexose-transporter genes were amplified by whole-cell PCR from strain FY1679 with GENEPAIRS primers obtained from Research Genetics. The *Arabidopsis thaliana STP2* and *STP3* ORFs were amplified from plasmids pUC19STP2 and pUC19STP3 using the primers F-ATSTP2 (5′-GGAATTCCAG CTGACCACCA TGGCTGTTGG TTCGATGA-3′), R-ATSTP2 (5′-GATCCCCGGG AATTGCCATG CTAGTCTTTG AAATATTTCT TCC-3′), F-ATSTP3 (5′-GGAATTCCAG CTGACCACCA TGGTAGCAGA AGAAGCAAG-3′) and R-ATSTP3 (5′-GATCCCCGGG AATTGCCATG TCAATGGCTA AGAATGGTG-3′), respectively. The *xylE* ORF was amplified from *Escherichia coli* strain JM109 with primers F-XYLE (5′-GGAATTCCAG CTGACCACCA TGAATACCCA GTATAATTCC-3′) and R-XYLE (5′-GATCCCCGGG AATTGCCATG TTACAGCGTA GCAGTTTGTT G-3′). PCR products were further amplified with primers T3 (5′-GAATAAACAC AAAAACAAAA AGTTTTTTTA ATTTTAAGGA ATTCCAGCTG ACCACC-3′) and T2-ORFs (5′-GGGGGAGGGC GTGAATGTAA GCGTGACATA ACTAATTACA TGACTCGAGG ATCCCCGGGA ATTGCCATG-3′). The predicted *A. thaliana* xylose-proton symporter ORF was amplified from a seedling cDNA bank (Minet *et al*., 1992) with primers F7-AT5G (5′-TAATTTTAAT CAAAAAATGT GTTTTAAATC GAGGCCTGAC-3′) and R-AT5G (5′-CCCCGGGAAT TGCCATGTCA CTTCAAGATT TTTGATTC-3′), and the PCR product was further amplified with primers T71-ORFs (5′-AACACAAAAA CAAAAAGTTT TTTTAATTTT AATCAAAAA-3′) and T2-ORFs. p4H7 was linearized with *Bam*HI and *Hin*dIII and transformed into yeast strain EBY.VW4000, together with the PCR-amplified sugar-transporter genes, selecting for uracil prototrophy on a medium containing 2% maltose after homologous recombination in yeast. Plasmids were reisolated, amplified in *E. coli* SURE (Stratagene), and analysed by restriction enzyme mapping or sequencing (Seqlab). The resulting plasmids were named pTH[name of gene] (e.g. pTHHXT1, pTHHXT3). Construction of plasmids YEpkHXT7, p426MET25-HXT11 and pHL125-GAL2 is described elsewhere (Krampe *et al*., 1998; Wieczorke *et al*., 1999; Liang & Gaber, 1996).

Other techniques. Molecular biology techniques were performed using published procedures (Sambrook *et al*., 1989). Xylose uptake was assayed as described previously (Weierstall *et al*., 1999). For the determination of the ethanol production rates, ethanol concentrations in yeast culture supernatants were measured using an enzymic test kit (Roche).

RESULTS

Yeast hexose transporters mediating uptake of xylose

The yeast strain TMB3001 (Eliasson *et al*., 2000), constructed by integrating a vector expressing *P. stipitis XYL*1 and *XYL2* (encoding xylose reductase and xylitol dehydrogenase, respectively) and *S. cerevisiae XKS*1 (encoding xylulose kinase) into the genome of an *S. cerevisiae* CEN.PK strain, grew slowly in shaken-flask cultures with synthetic medium and 2% xylose as the carbon source (Fig. 1). In contrast, strain TMB3201, constructed by integrating the same plasmid into the CEN.PK-derived strain EBY.VW4000 (Wieczorke *et al*., 1999) lacking all its hexose-transporter genes, did not grow at all in the same medium. This demonstrates that one or more of the yeast hexose transporters is responsible for the uptake of xylose in *S. cerevisiae*.

In order to find the transporter(s) responsible, the *S. cerevisiae* hexose-transporter genes *HXT1, 3, 4, 5, 8, 9, 10, 13, 14* and 15 were cloned by recombination-cloning into the multicopy expression vector p4H7, driving expression from the very strong and constitutive *HXT7*$^{1–392\ bp}$ promoter fragment. The resulting plasmids, the empty vector, and plasmids YEpkHXT7, p426MET25-HXT11 and pHL125-GAL2 (expressing the *S. cerevisiae* Hxt7, Hxt11 and Gal2 transporters, respectively), were transformed into the yeast strain TMB3201. The cells were plated onto agar plates of synthetic medium without uracil, containing 2% maltose as the carbon source. After replica-plating, all the transformants except those with the empty vector could grow on synthetic medium with glucose as the sole carbon source, indicating that all transporters were functionally expressed and able to mediate uptake of sugars into the cells.

To identify which hexose transporters are able to take up xylose, the growth rates of the transformants in liquid synthetic medium without uracil and with 2% xylose as the sole carbon source were determined. Only

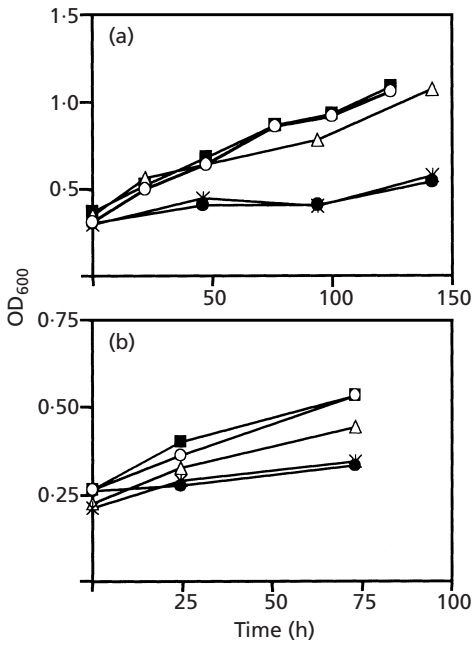

Fig. 1. Growth of *HXT* mutant strains in xylose medium. Yeast transformants were pregrown in synthetic medium without uracil and with 2% maltose, washed twice and inoculated in synthetic medium without uracil and with 2% xylose at an initial OD_{600} of about 0·35 (a) or 0·25 (b). Cells were grown aerobically at 30 °C on a rotary shaker. Growth was monitored by measuring the OD_{600} of the cultures. Strains are: (a and b) TMB3001(p4H7) (■), TMB3201(p4H7) (●); (a) TMB3201(pTHHXT1) (×), TMB3201(YEpkHXT7) (△), TMB3201(pHL125-GAL2) (○); (b) TMB3201(pTHHXT4) (○), TMB3201(pTHHXT5) (△), TMB3201(pTHHXT9) (×). Representative results of at least two independent experiments for each strain are shown.

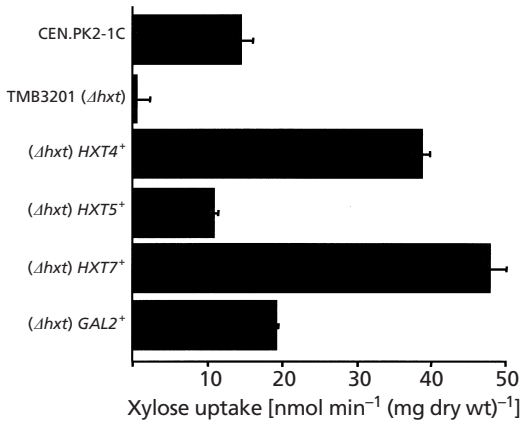

Fig. 2. Xylose uptake in *HXT* mutant strains. Yeast transformants CEN.PK2-1C(p4H7), TMB3201(p4H7), TMB3201(pTHHXT4), TMB3201(pTHHXT5), TMB3201(YEpkHXT7) and TMB3201(pHL125-GAL2) were grown into the exponential growth phase in synthetic medium without uracil and with 2% maltose before harvesting. Xylose uptake was measured for 1 min with 50 mM D-[U-^{14}C]xylose. The results are means of three determinations ± SEM.

transformants expressing the Hxt4, Hxt5, Hxt7 or Gal2 transporters were able to grow in the xylose medium (Fig. 1). In contrast, transformants expressing the transporters Hxt1, 3, 8, 9, 10, 11, 13, 14 and 15 did not grow with 2% xylose. Our results indicated that Hxt4, 5 and 7, and Gal2, are able to mediate the uptake of xylose into *S. cerevisiae* cells. We did not analyse Hxt2 as it was not possible to clone it behind the *HXT7* promoter fragment on vector p4H7. Hxt6, Hxt16 and Hxt17 were not included because they are almost identical to Hxt7, Hxt15 and Hxt13, respectively. *HXT12* is a pseudogene (Wieczorke *et al*., 1999).

Determination of xylose-uptake activities

To confirm the results obtained in the growth tests, short-term (60 s) xylose uptake assays were performed with radiolabelled xylose. Xylose uptake was linear for at least 60 s under the conditions used (data not shown). Transformants of strain TMB3201 expressing Hxt4, Hxt5, Hxt7 or Gal2, or containing the empty vector p4H7, were grown into the exponential growth phase in synthetic medium without uracil and with 2% maltose. As expected, the hexose-transport-deficient strain TMB3201 with the empty vector was not able to take up xylose (Fig. 2). In contrast, expression of Hxt4, 5 and 7, and Gal2, conferred xylose uptake activities upon the strain. Xylose uptake activity with 50 mM radiolabelled xylose was highest for the strain expressing Hxt7 [48 nmol min^{-1} (mg dry weight)$^{-1}$] and lowest for the strain expressing Hxt5 (11 nmol min^{-1} (mg dry weight)$^{-1}$] (Fig. 2). The parental strain CEN.PK2-1C transformed with the empty vector p4H7 exhibited an uptake activity of 15 nmol min^{-1} (mg dry weight)$^{-1}$.

Expression of heterologous monosaccharide transporters in *S. cerevisiae*

The *S. cerevisiae* hexose transporters solely mediate facilitated diffusion of their substrates down a concentration gradient (Lagunas, 1993). In contrast, several other yeasts, plant cells and bacterial cells can actively transport hexoses and pentoses by proton-symport mechanisms against concentration gradients (Boles & Hollenberg, 1997; Büttner & Sauer, 2000). Moreover, these proton-symport systems normally exhibit much higher affinities for their substrates. We therefore investigated whether such heterologous transporters could restore growth on xylose to the monosaccharide-transport-deficient yeast strain TMB3201.

The *E. coli xylE* gene encodes a xylose–proton symporter (Davis & Henderson, 1987). The hexose–proton symporters Hup1 from *Chlorella kessleri*, and Stp2 and Stp3 from *A. thaliana*, have been shown to mediate uptake not only of various hexoses but also of xylose (reviewed by Büttner & Sauer, 2000). Moreover, after completion of the *A. thaliana* genome sequencing project, a sequence has been found on chromosome 5 encoding a predicted xylose–proton-symporter-like protein targeted to chloroplasts (accession number At5g59250). The *xylE*, *STP2* and *STP3* ORFs, as well as

the putative xylose–proton symporter sequence lacking the 31 amino acids encoding the chloroplast-targeting sequence, were cloned into the multicopy expression vector p4H7. After transformation of the resulting plasmids and plasmid pNEV-E expressing *HUP1* behind the yeast *PMA1* promoter (Robl *et al*., 2000) into strain TMB3201, Stp2, Stp3 and Hup1, but not – as expected – XylE and the putative *A. thaliana* xylose–proton symporter, supported growth of the yeast transformants on synthetic medium with 2 % glucose (data not shown). However, none of the transporters restored growth in synthetic medium with 2 % xylose, indicating that they are not able to take up xylose into the yeast cells. Moreover, we transformed strain TMB3201 with the *P. stipitis* genomic DNA library YEpTW (Weierstall *et al*., 1999) and an *A. thaliana* seedling cDNA bank (Minet *et al*., 1992), and selected the transformants for growth in synthetic medium with 2 % xylose. From a total of more than 18000 and 10000 transformants, respectively, we could not find any transformant able to utilize and to grow on xylose.

Xylose fermentations and growth tests with *HXT*-overexpressing strains

The *S. cerevisiae* PUA strains were selected for faster growth on xylulose media (Porep, 1987; Kötter & Ciriacy, 1993). Subsequently, the *P. stipitis XYL1* and *XYL2* ORFs under control of the *S. cerevisiae PDC1* and *ADH1* promoters, respectively, were chromosomally integrated in these strains, resulting in PUA-X1,2. In order to see whether overexpression of xylose-transporting proteins could increase the ethanol production rate from xylose by recombinant *S. cerevisiae* strains, pTHHXT5, YEpkHXT7, pHL125-GAL2 and the empty vector were transformed into the PUA-X1,2 strain. The transformants were pre-grown on synthetic medium with 2 % xylose to an OD_{600} of 1·9. Cells were harvested by centrifugation and washed twice with 2 % xylose medium. They were then resuspended in 0·1 vol. of the volume of the same medium, incubated at 30 °C and the ethanol concentration in the medium determined at different time points. As can be seen in Fig. 3, overexpression of the monosaccharide transporters did not increase the ethanol production rate. Instead, it seemed that ethanol production was even slightly delayed, especially in the case of the Gal2-overproducing transformants.

Under anaerobic conditions, the recombinant PUA strains cannot grow on xylose medium (Kötter & Ciriacy, 1993; data not shown). Therefore, the growth rates of the transformants were determined under aerobic conditions in shaken-flask cultures with synthetic medium and 2 % xylose. Growth on xylose of the recombinant PUA strains was twice as fast as that of the CEN.PK-derived strains (doubling times about 13 and 29 h, respectively). However, the growth rates of the transformants overexpressing the xylose-transporting proteins Hxt4, Hxt7 and Gal2 were the same as those of the transformants with the empty vector. Moreover, no ethanol could be detected in the culture supernatants of any of the transformants. Our results indicate that xylose uptake does not determine xylose flux, at least not as the only step in xylose utilization.

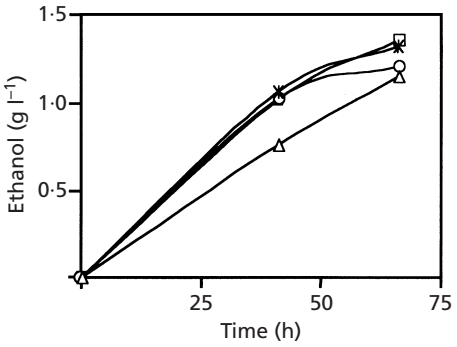

Fig. 3. Ethanol production of PUA-X1,2 cells overexpressing xylose transporters. Transformants TMB3001(p4H7) (×), TMB3201(pTHHXT5) (□), TMB3201(YEpkHXT7) (○) and TMB3201(pHL125-GAL2) (△) were pregrown in synthetic medium without uracil and with 2 % xylose to an OD_{600} of 1·9, washed twice, resuspended in 0·1 vol. of the same medium, and incubated under (semi-)anaerobic conditions at 30 °C. Ethanol concentrations in the culture supernatants were determined at various time points. Representative results of at least two independent experiments for each strain are shown.

DISCUSSION

We have shown that after deletion of all of the 18 hexose-transporter genes from a recombinant yeast strain able to utilize the pentose sugar xylose, its ability to take up and to grow on xylose is lost. This is, for the first time, a genetical proof that the yeast hexose-uptake system also mediates uptake of xylose. It confirms earlier results obtained by kinetic and physiological analysis of xylose uptake in yeast cells (Serrano & de la Fuente, 1974; Busturia & Lagunas, 1986; Kötter & Ciriacy, 1993; Meinander & Hahn-Hägerdal, 1997). After overexpression of individual hexose-transporter genes in the xylose uptake-deficient yeast strain, we could demonstrate that at xylose concentrations of 2 % only Hxt4, Hxt5, Hxt7 and Gal2 are able to transport the sugar in significant amounts. Hxt7 and Gal2 are high-affinity hexose transporters whereas Hxt4 and Hxt5 are moderately low in affinity for glucose (Reifenberger *et al*., 1997; Diderich *et al*., 2001). We have not tested Hxt2 and Hxt6. However, as Hxt6 is nearly identical to Hxt7, and Hxt2 shows transport kinetics similar to Hxt4, we propose that also these transporters can mediate uptake of xylose. However, the low-affinity hexose transporters Hxt1 and Hxt3, and also the normally very weakly expressed transporters Hxt8 to Hxt17 were not able to transport xylose, at least in amounts sufficient to support growth.

The results were obtained after constitutive overexpression of the hexose-transporter genes. Normally, these genes are regulated by the presence and concentration of glucose or galactose (reviewed by Boles & Hollenberg, 1997; Özcan & Johnston, 1999). Whereas expression of

HXT1 to *HXT4* requires the presence of glucose in the medium, *GAL2* is only expressed in the presence of galactose and absence of glucose. In contrast, *HXT5* and *HXT7* are also expressed in the absence of glucose (Schulte *et al*., 2000; Diderich *et al*., 1999, 2001). This means that in recombinant xylose-utilizing *S. cerevisiae* cells during growth on xylose mainly Hxt5 and Hxt7 may contribute to the uptake of the sugar.

None of the heterologous sugar transporters was able to support growth on xylose. In the case of *E. coli* XylE and the predicted *A. thaliana* xylose–proton symporter, we cannot exclude that these transporters are not correctly targeted to the plasma membrane in a functional form, as has been observed for several heterologous membrane proteins in yeast (Villalba *et al*., 1992; Jahn *et al*., 2002; our own unpublished results). However, *C. kessleri* Hup1 and *A. thaliana* Stp2 and Stp3 were functionally expressed in the plasma membrane, as they supported growth of the hexose-transport-deficient yeast strain on glucose. Growth of these strains on glucose was significantly lower than parental growth, indicating that the capacity of the heterologous transporters was very low. For all three transporters, xylose-uptake activity has been demonstrated (Büttner & Sauer, 2000). Therefore, we propose that the xylose-uptake activity mediated by Hup1, Stp2 and Stp3 is too low to support growth on xylose.

Overexpression of the xylose-transporting hexose transporters in a xylose-utilizing *S. cerevisiae* PUA strain did not result in faster growth on 2% xylose medium under aerobic conditions, nor did it result in an increase of the ethanol production rate under (semi-)anaerobic conditions. This is in contrast to the recent observation that under anaerobic conditions in chemostat cultures with mineral medium increasing the xylose concentration in the feed enhanced the xylose flux, suggesting that transport limits flux (Eliasson *et al*., 2000). However, in that study a different, CEN.PK-derived yeast strain was used. Indeed, the PUA strain that we have used has been selected for faster growth on xylulose medium (Porep, 1987; Kötter & Ciriacy, 1993). One of its new properties is a higher endogenous level of xylulose kinase activity. The growth rate on xylose was more than twofold higher for the PUA-derived recombinant strain than for the CEN.PK-derived strain. Interestingly, the xylose-uptake activity was also about twofold higher in PUA cells (data not shown). This could mean that in the PUA strain during selection for faster growth on xylulose the sugar-uptake system was one of the steps that were optimized. Unfortunately, the parental strain is no longer available to confirm this assumption. Nevertheless, it seems that in different yeast strains different factors might contribute to limited xylose fermentation, with xylose uptake being one of them.

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