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Anaerobic growth and improved fermentation of *Pichia stipitis* bearing a *URA1* gene from *Saccharomyces cerevisiae*

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Abstract Respiratory and fermentative pathways coexist to support growth and product formation in *Pichia stipitis*. This yeast grows rapidly without ethanol production under fully aerobic conditions. and it ferments glucose or xylose under oxygen-limited conditions. but it stops growing within one generation under anaerobic conditions. Expression of *Saccharomyces cerevisiae URA1 (ScURA1)* in *P. stipitis* enabled rapid anaerobic growth in minimal defined medium containing glucose when essential lipids were present. *ScURA1* encodes a dihydroorotate dehydrogenase that uses fumarate as an alternative electron acceptor to confer anaerobic growth. Initial *P. stipitis* transformants grew and produced 32 g/l ethanol from 78 g/l glucose. Cells produced even more ethanol faster following two anaerobic serial subcultures. Control strains without *ScURA1* were incapable of growing anaerobically and showed only limited fermentation. *P. stipitis* cells bearing *ScURA1* were viable in anaerobic xylose medium for long periods. and supplemental glucose allowed cell growth. but xylose alone could not support anaerobic growth even after serial anaerobic subculture on glucose. These data imply that *P. stipitis* can grow anaerobically using metabolic energy generated through fermentation but that it exhibits fundamental differences in cofactor selection and electron transport with glucose and xylose metabolism. This is the first report of genetic engineering to enable anaerobic growth of a eukaryote.

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Introduction

Most yeast and fungi are Crabtree-negative, which is to say that they lack the ability to grow anaerobically, and must rely on respirofermentative metabolism to support cell growth (Siso et al. 1996). Molecular oxygen acts as a terminal electron acceptor of respiratory metabolism. and it is required for biosynthesis of membrane sterols in yeasts (Gancedo and Serrano 1989). However. rare species such as *Saccharomyces cerevisiae* can grow anaerobically using energy solely generated from fermentation when essential lipids are present (Andreasen and Stier 1953). Nagy et al. (1992) showed that *S. cerevisiae* possesses a unique form of dihydroorotate dehydrogenase (EC 1.3.3.1) that confers the ability to grow anaerobically.

Dihydroorotate dehydrogenase. encoded by *ScURA1* (Roy 1992), catalyses a single redox reaction converting dihydroorotate to orotate in the pyrimidine biosynthesis pathway. Dihgydroorotate dehydrogenases of higher eukaryotes are functional components of the respiratory chains. using oxygen as the ultimate (Vorisek et al. 1993). but not necessarily the only, electron acceptor (Hines et al. 1986). In *S. cererisiae*, the enzyme is found in the cytosol where its activity is also coupled to the reaction that reduces fumarate to succinate (Nagy et al. 1992).

S. cererisiae is incapable of using xylose because it lacks two key xylose-metabolizing enzymes, xylose reductase (*PsXYL1*) and xylitol dehydrogenase (*PsXYL2*). However, this yeast can ferment xylulose (Yu et al. 1995) because of the presence of a xylulokinase (*ScXUK*) (Ho and Chang 1989). Recently. *XYL1* and *XYL2* genes from the xylose-fermenting yeast, *Pichia stipitis*, have been used to impart xylose fermentation to *S. cerevisiae* (Kotter and Ciriacy 1993; Tantirungkij et al. 1994; Meinander et al. 1996) but the resulting strains have failed to ferment xylose effectively. Toon et al. (1997) improved the *Saccharomyces* xylose fermentation by generating a fusion strain that overexpresses *PsXYL1*.

PsXYL2 and *ScXUK*. An alternative approach. which is presented in this study. is to introduce useful genes from *S. cerevisiae* into *P. stipitis* to make the latter a better fermenter. *P. stipitis* is one of the best-studied xylose-fermenting yeasts because it produces high ethanol yields under low oxygen levels (Ligthelm et al. 1988b; du Preez et al. 1989). Moreover, its mating (Melake et al. 1996) and transformation (Yang et al. 1994; Lu et al. 1998) systems have been developed in recent years. *P. stipitis* differs from *S. cerevisiae* in respiratory and fermentative metabolism. Glucose induces fermentation (Chambers et al. 1995; Trumbly 1992) and represses respiration (Lagunas 1986) in *S. cerevisiae*, but this sugar does not play similar roles in *P. stipitis*. Instead, a low oxygen level induces fermentative enzymes in *P. stipitis*, and respiration is not repressed by fermentable sugars (Passoth et al. 1996; Skoog and Hahn-Hägerdal 1990). Moreover, *P. stipitis* possesses an alternative cyanide-insensitive, salicyl hydroxamate-sensitive electron-transport system that is not found in *S. cerevisiae* (Jeppsson et al. 1995).

The dependence of *P. stipitis* on oxygen for growth and its constitutive respiratory activity diminish ethanol yields. Moreover. the requirement for controlled aeration increases process and control costs. We therefore engineered *P. stipitis* by introducing the *URA1* gene from *S. cerevisiae*. Our goal was to eliminate the oxygen requirement for growth and thereby increase the ethanol yield from mixed sugars. Second. we hoped to gain a powerful screening tool for future strain selections. Here we report a genetically engineered *P. stipitis* strain that is able to grow anaerobically. However, the capacity for anaerobic growth was limited to glucose. and unrestricted anaerobic growth was not obtained on xylose. These findings indicate that additional factors might limit anaerobic growth and fermentation of *P. stipitis* on xylose, and they also show that imparting the capacity for anaerobic growth to eukaryotes can be a simple matter if the organism is capable of generating energy by fermentation.

Materials and methods

Strains and transformation

Escherichia coli XL1 Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 SupE44 relA1 lac [F'proAB laclq ZDM 15 Tn10 Ter']*) was used as the bacterial host for plasmids (Stratagene). Competent cells were produced and transformed as described in Sambrook et al. (1989). *Pichia stipitis* FPL UC7 (*ura3-3*) (NRRL Y-21448). which was derived from FPL DX26 (NRRL Y-21304) (Lu et al. 1998). and FPL TJ26 (*ura3*-1) (Yang et al. 1994). which was derived directly from CBS 6054. were used as the recipient hosts for yeast transformation. A wild-type *S. cerevisiae* strain. 288 C (Polaina and Wiggs 1983) (*MATa SUC2 mal gal2 CUP1*) was used as a control in the anaerobic growth experiment on glucose. *P. stipitis* transformation was carried out by the lithium acetate method (Rose et al. 1990).

Media and growth conditions

Escherichia coli XL1 Blue was gown on Luria-Bertani medium at 37°C and 50 µg/ml ampicillin was added to the medium for selection of transformants. Yeast strains were routinely cultivated at 30°C in yeast/peptone/glucose medium containing 10 g/l yeast extract. 20 g/l peptone and 20 g/l glucose. Yeast transformants were grown on minimum medium containing 1.7 g/l yeast nitrogen base without amino acids (Difco), 5 g/1 $(NH_4)_2SO_4$. and 20 g/l glucose. Uridine was added at 20 mg/l when required for growth of the parental strain. FPL UC7 (*ura3-3*). The components for the anaerobic growth medium comprised 1.7 g/l yeast nitrogen base, 2.27 g/l urea, 6.56 g/l peptone, and 80 g/l glucose or xylose. Yeast strains were pre-cultured on yeast nitrogen base (YNB)/glucose plates for 2 days. Cells were then inoculated into 25 ml fermentation medium in a 50-ml conical flask. Cultures were shaken at 100 rpm at 30°C for 24 h; they were then harvested and washed with sterile water and used as inocula for the anaerobic growth experiments. Anaerobic fermentations were performed in 40-ml serum vials each containing 25 ml medium. We stopped the vials with rubber bungs and crimped aluminum seals. and flushed each with argon gas for 15 min before autoclaving. A lipid mixture consisting of ergosterol. Tween 80 and linoleic acid (5 ml/l; Jessens et al. 1983) plus 0.2 mM fumarate and inoculum was injected into each viaI using a 10-ml syringe and an 18.5-gauge needle. To eliminate residual oxygen, Oxyrase (Qxyrase Inc.) was injected into each vial at 0.5 ml/25 ml. Cultures were then shaken at 100 rpm at 30°C. For anaerobic growth experiments on agar plates, the same lipid-containing. Oxyrase-containing medium was used in an OxyDish.

Plasmid construction

Restriction and other DNA-modifying enzymes were purchased from Promega or New England Biolabs. Oligonucleotide primers were synthesized by Ransom Hill Inc. and Genosys Inc. DNA standards were obtained from Gibco BRL. The *E. coli/P. stipitis* shuttle vector pjM6 (Yang et al. 1994) was used as the parental plasmid for the expression cassettes. To coustruct pNQ17, a 630-bp *P. stipitis* xylose reductase terminator fragment was first amplified by primer 1: **5'-GGAATTCGATCCACAGACACTAATTG-3'** and primer 2: **5'-TCTAACATTGTAGTATAGTTGTATAGAG-**3′ from a pXOR plasmid (Dahn et al. 1996) and cloned into pJM6 as pNQ12. A 598-bp *P. stipitis* alcohol dehydrogenase 1 ***(PsADH1)*** promoter (Cho and Jeffries 1998) was amplified with primer 3: 5′-**TGCACTGCAGGATCCGAGGGAAAAC-3'** and by primer 4: **5'-GATAATTTGGATGGATCGCAGCAC-3'**. A 944-bp *S. cerevisiae URA1* gene was amplified with primer 5: **5'-AACAATGA-CAGCCAGTTTAACTACCAAG-3'** and primer 6: **5'-GGGGG-ATCCTTAATGCTGTTCAACTTCCC-3'** from *S. cerevisiae* 288C genomic DNA. Therefore, a 1542-bp fusion of ***ADH1$_P$-URA1*** was created by using primers 3 and 6 following the method of Ali and Steinkasserer (1995) and excised as a *Pst*I/*Bam*HI fragment. This fragment was then ligated into the *Pst*I/*Bam*HI sites of pNQ12 to create pNQ17. The size of the expression cassette containing ***ADH1$_P$-URA1-XYL1$_T$*** **was 2172 bp.**

DNA manipulation

Putative transformants were picked and grown on YNB/glucose minimal medium (Rose et al. 1990). Plasmid DNA was isolated using Qiagen spin columns (Qiagen). Genomic DNA was isolated following the method of Rose et al. (1990) and 20 µg was loaded on each lane Southern hybridization was performed according to Sambrook et al. (1989) to confirm the putative transformants. DNA was transferred to a Nytran filter (Schleicher & Schü) and probed with a 944-bp *ScURA1* gene labeled with digoxigenin by the Genius 1 kit (Boehringer Mannheim). Genomic DNA was digested with *Pst*I and *Bam*HI restriction enzymes. Hybridization was performed in 25% formamide at 37°L.

Sampling and analysis

Samples of 1 ml were withdrawn daily from each vial using 1 ml syringes equipped with 22-gauge needles. Cell growth was determined by monitoring alasorbance at 600 nm, and converting to dry weight (Cho and Jeffries 1998). Under the experimental conditions. as A_{600} of 1.0 equals 0.24 g/1 cells. Then the samples were spun for 5 min at 14 000 rpm. The supernatant solutions were used for HPLC or GC analysis to determine the sugar consumption and ethanol production rates (Jeffries 1982).

Results

Introduction of *ScURA1* into *P. stipitis*

We constructed an expression cassette (pNQ17) based on pJM6 (Yang et al. 1994) from *P. stipitis* by fusing the *PsADHI* (Cho and Jeffries 1998) promoter to the *ScURA1* gene (Fig. 1A) and the *PsXYL1* terminator (Dahn et al. 1996) and we confirmed the construct by restriction mapping and sequencing. Putative transformants were cultivated on YNB/glucose minimal medium and confirmed by genomic Southern hybridizations (Fig. 1B). The positive control, genomic DNA from wild-type *S. cerevisiae* 288C (Polaina and Wiggs 1983). showed a 4.3-kb band when probed with the *ScURA1* fragment. A 1.54-kb fragment was detected in the genomic DNA from four individual UC7 (pNQ17) transformants. This corresponded to the pNQ17 plasmid control and indicated that these strains carried the *ScURA1* gene. The *P. stipitis* genomic DNA showed no cross-hybridization with the *ScURA1* gene under the conditions employed, suggesting that the *PsURA1* gene differs significantly from its *S. cerevisiae* homolog. Transformation of *P. stipitis* UC7 (*ura3-3*) with pJM6 was confirmed by Southern hybridization with a *PsURA3* probe. and the transformant was used as a negative control (data not shown).

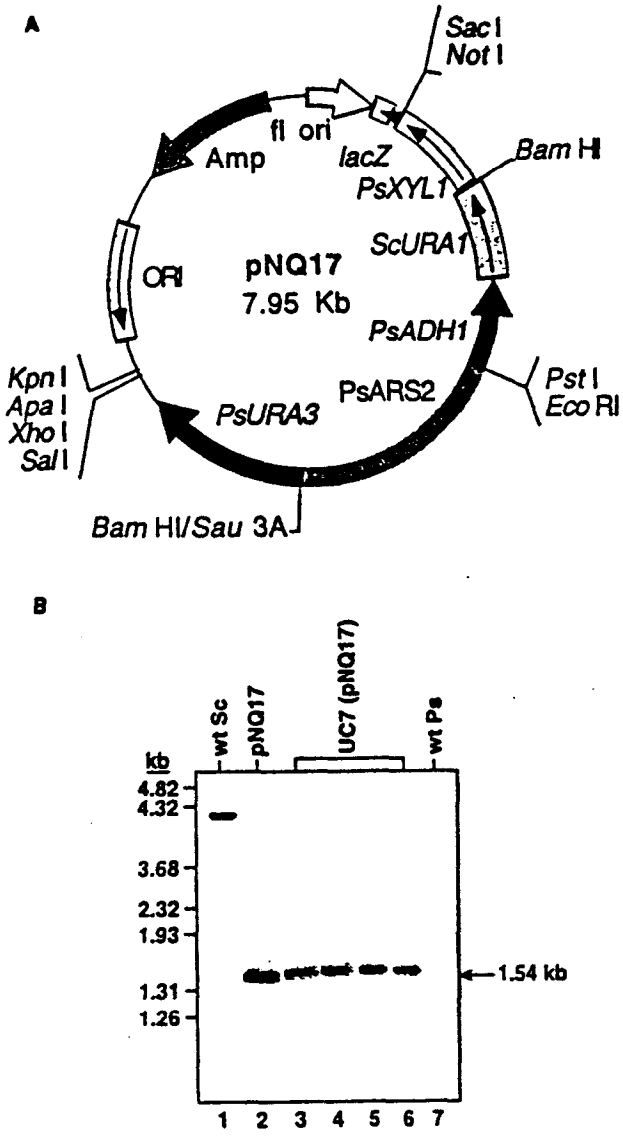

Fig. 1 A Plasmid map of pNQ17 that contains the 2172-bp $PsADH1_P$-*ScURA1*-$PsXYL1_T$ expression cassette. The cassette was subcloned in a high-copy-number autonomous vector of *Pichia stipitis* using *PsURA3* as the selectable marker. **B** Southern analysis of the putative UC7(pNQ17) transformants. Lanes: *1 Sacharomyces cerevisiae* 288C genomic DNA (positive control), *2* plasmid pNQ17 control (100 ng DNA); *3-6* genomic DNA isolated from four individual putative transformants of UC7(pNQ17). *7* wild-type *P. stipitis* CBS 6054 genomic DNA All the DNA was digested with *Pst*I and *BamH*I restriction enzymes. The probe was a polymerase-chain-reaction-amplified 944-bp *ScURA1* gene labeled with Digoxigenin. The DNA molecular mass marker used was *Bst* EII-digested lambda DNA. *Arrow* the expected size of the *ADH1* promoter and the *URA1* gene

Rapid anaerobic growth and fermentation on glucose

P. stipitis UC7(pNQ17) transformants were used to test anaerobic growth and fermentative capacities on glucose. In our preliminary trial. anaerobic growth of UC7(pNQ17) was observed-on- glucose after a lag of 93 h. The addition of fumarate (0.2 mM) shortened the lag phase. so we added it to all cultures in subsequent trials. With fumarate present, UC7(pNQ17) started growing after 41 h in the initial set and increased from 0.08 of cells 1 to 6.6 g cells/l within 73 h. At 93 h UC7(pNQ17) reached 7.5 g cells/l. corresponding to a 94-fold increase in cell mass (Fig. 2A1). Transformants produced 32 g ethanol/l from 78 g glucose within 73 h (Fig. 2B1, CI). This was the first time that anyone had enabled *P. stipitis* to grow anaerobically. The negative control UC7(pJM6) as well as the parental strain UC7 stayed near their initial cell densities of 0.34-0.41 g cells/1 and consumed little glucose within the 5 days of fermentation. The *S. cerevisiae* 288 C strain started growing within 17 h and reached 8.5 g cells/l at 93 h. indicating that conditions were ideal for its anaerobic growth. To ensure that the capacity for anaerobic growth was not specific to the UC7 strain, we transformed another *P. stipitis* host. TJ26, with pNQ17 or pJM6. The transformants were cultivated on OxyDish

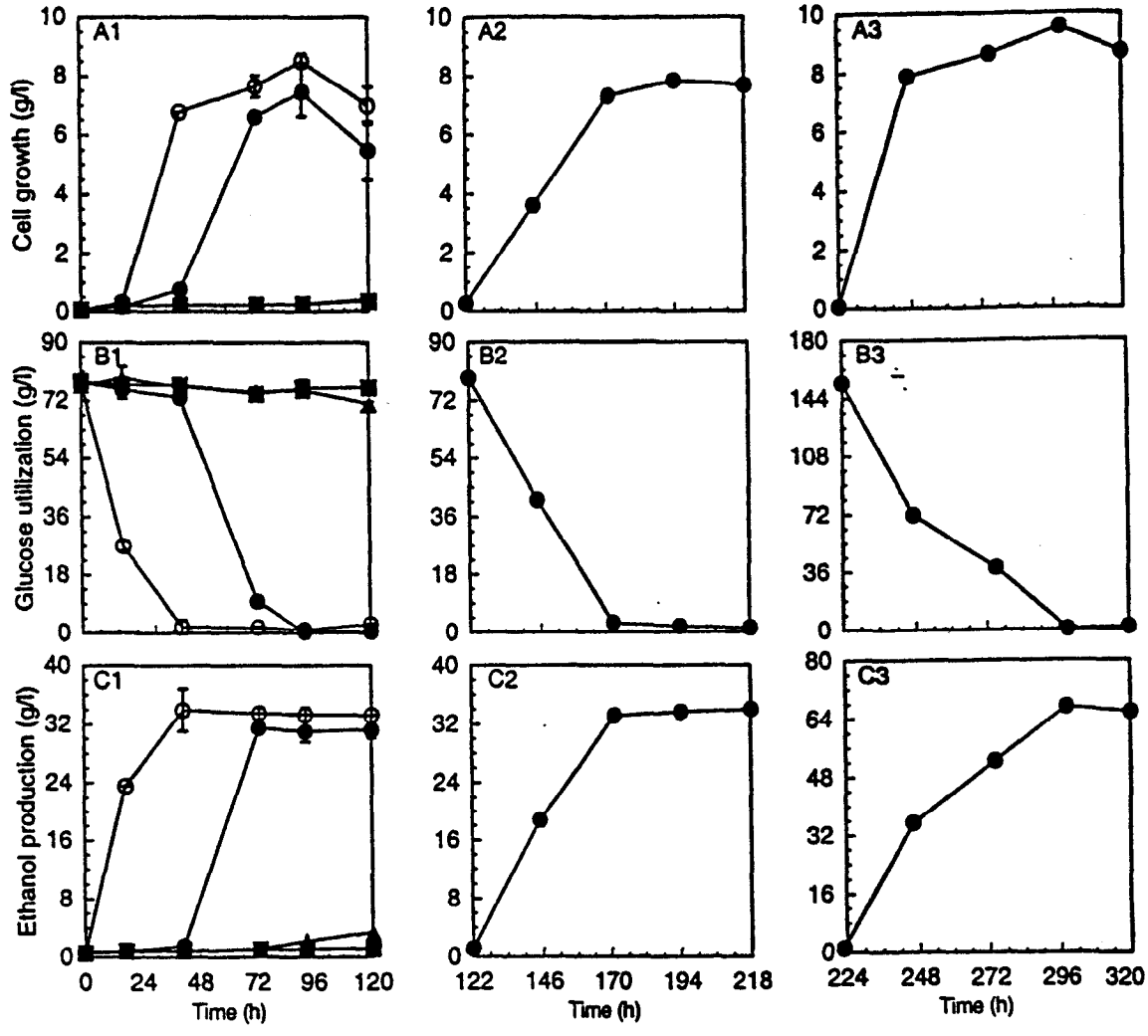

Fig. 2A-C The anaerobic growth experiment of the UC7(pNQ17) strain on glucose. **A1, B1, C1,** Cell growth, glucose utilization and ethanol production from the initial set respectively. There are three replicates in the experiment and the starting glucose concentration was 78 g 1, ○ *S. cerevisiae* 288 C. ● *P. stipitis* UC7(pNQ17). ■ *P. stipitis* UC7(pJM6).▲ FPL UC7 supplemented with 20 mg 1 uridine. Two subsequent sets of anaerobic serial subcultures in 79 g/l **(A2, B2, C2)** and 154 g l (A3, B3, C3) glucose are shown. The data plotted in the subculture experiment are from duplicate cultures

plates containing the same components as in the liquid medium. We observed confluent growth from TJ26(pNQ17) on glucose within 3 days. but we observed little or no growth from the TJ26(pJM6) control.

Serial subculture of *P. stipitis* transformants on glucose

We subcultured cells of UC7(pNQ17) to confirm our results from the initial trial. Inocula cultivated anaerobically on glucose for 120 h were aseptically transferred in duplicate to fresh anaerobic culture medium at 122 h. The UC7(pNQ17) strain bearing *ScURA1* started growing within 23 h and increased from 0.28 g cells/l to 7.3 g cells/l within 49 h (Fig 2A2). In addition. strain UC7(pNQ17) produced 33 g ethanol/1 from 79 g glucose/l (Fig. 2B2, C2). Cells from the second set of UC7(pNQ17) cultures were transferred to a third set of fresh anaerobic media containing 154 g glucose/l at 224 h. The cells started growing even more rapidly and went from 0.07 g cells/l to 7.9 g cells/l within 17 h. (Fig. 2A3). Cultures used up the glucose within 75 h and produced 67 g ethanol/l (Fig. 2B3, C3). These data indicated that this engineered strain can ferment glucose to a readily recoverable ethanol concentration.

Anaerobic growth and fermentation on xylose

In contrast to its performance on glucose. the UC7(pNQ17) strain did not exhibit elevated anaerobic growth on xylose. The cell mass of UC7(pNQ17) doubled only once and consumed only 7.9 g xylose/l after 120 h (Fig. 3A, B). These observations agreed with the previous report by du Preez et al. 1989 that *P. stipitis* is incapable of anaerobic growth. Anaerobic growth of

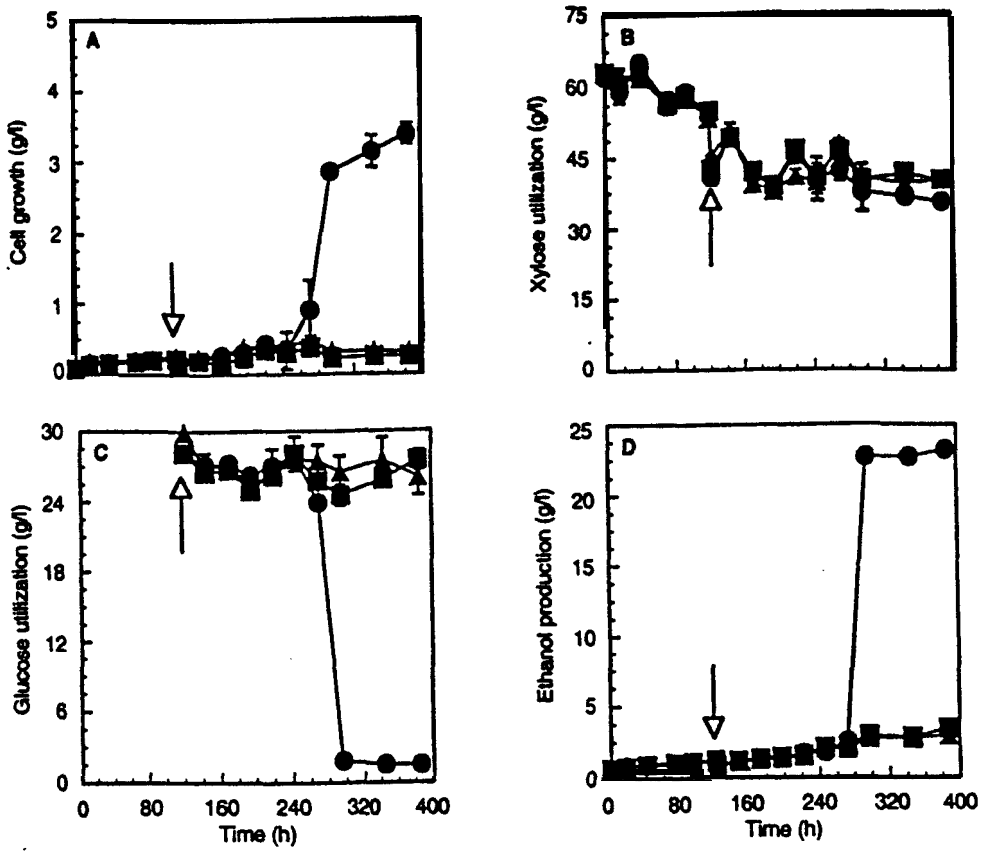

Fig. 3A-D Lack of anaerobic growth of UC7(pNQ17) on xylose. There are three replicates in the experiment. A Cell growth a 62 g l xylose plus an additional 48 g l glucose. **B** Xylose utilization. **C** Glucose utilization. **D** Ethanol production from xylose and glucose. *Arrow* time (122 h) when the additional glucose was injected into all cultures. ● UC7(pNQ17): ■ UC7(pJM6) containing the parental plasmid only, ▲ FPL UC7 supplemented with 20 mg 1 uridine

UC7(pNQ17) was not significantly different from that of the UC7(pJM6) control or the UC7 parental strain. Likewise. the TJ26(pNQ17) transformant did not grow anaerobically on the xylose OxyDish.

We did not expect to observe such a difference in the performance of the *ScURA1* transformants on xylose and glucose under otherwise similar conditions. so we added 48 g 1 glucose to the liquid cultures at 122 h to see if it would enable the cells to use xylose. The UC7(pNQ17) cultures started growing 88 h after glucose addition. This delay was similar to what we observed following the first inoculation to glucose without xylose. The cell mass of UC7(pNQl7) increased from 0.15 g cells-ml to 3.33 g cel1s.l while the UC7(pJM6) and the UC7 parental strains stayed at 0.25-0.31 g cells/1 (Fig. 3A). Even though UC7(pNQ17) consumed almost all the additional glucose within 385 h (Fig. 3C). it used only 5.3 g xylose 1 (Fig. 3B). Transformed cells produced 23 g ethanol/1 compared to only 3-3.4 g/l from the controls (Fig. 3D). These data showed that growth and ethanol production occurred mainly in response to the additional glucose. The addition of glucose to the xylose medium did not enable anaerobic growth on xylose UC7(pNQ17).

In a parallel experiment. we cultivated UC7(pNQ17) anaerobically for two passages on glucose then shifted the cells to a medium containing 58 g xylose/l to determine whether pre-induction for anaerobic growth on glucose would impart anaerobic growth on xylose. At the same time some of these cells were also transferred to media containing 78 g/l glucose. After 150 h on the xylose medium. UC7(pNQ17) transformants underwent only one doubling (Fig. 4A). Only 7 g xylose/l was consumed and 1.2 g ethanol/l was produced by each strain after 150 h (Fig. 4B, C). In contrast. the control that we had transferred to glucose produced 34 g ethanol/l (Fig. 4D) within a short time. From these results. it appears that introducing *ScURA1* into *P. stipitis* is not sufficient to enable it to grow anaerobically on xylose. This finding suggests that other unknown factors limit the anaerobic growth of *stipitis* on xylose.

Discussion

We cloned the *ScURA1* gene of *S. cerevisiae* and successfully transferred it into *P. stipitis.* which then showed improved capacities for anaerobic growth and elevated fermentation on glucose. The engineered strain performed even better following serial subcultures. This suggests that some adaptation occurred during the lag phase. We previously observed an increase in copy number with the pJM6 vector (upon which pNQ17 is based) when we introduced a *PsURA3* gene into *P. stipitis* TJ26 *(ura3-1).* After 50 passages in selective

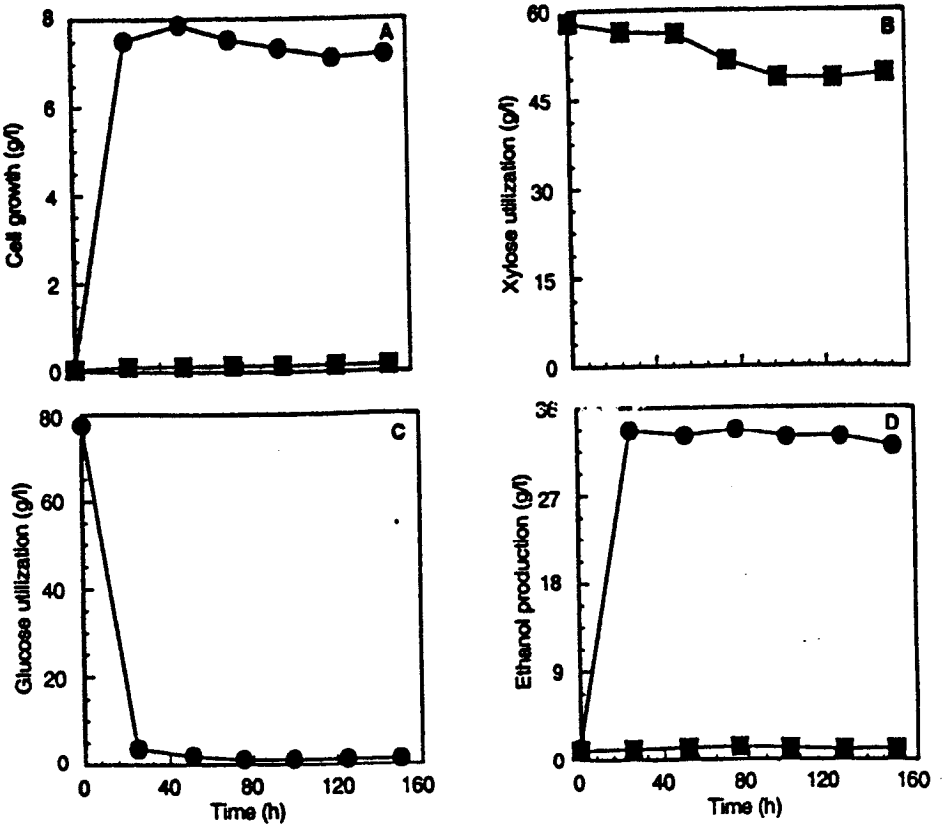

Fig. 4A-D Anaerobic culture of UC7(pNQ17) transferred from glucose to xylose. ■ UC7(pNQ17) after two passages on glucose transferred to 58 g l xylose: ● UC7(pNQl7) after two passages on glucose transferred to 150 g/l glucose. A Cell growth. **B** Xylose utilization. **C** Glucose utilization. **D** Ethanol production on xylose or glucose. Data plotted are from duplicates in the experiment

medium. the pJM6 copy number increased from about 1 to about 15 copies/cell (Yang et al. 1994). It is possible that the copy number of pNQ17 increased in the subculture experiment on glucose. which resulted in rapid anaerobic growth.

In contrast to the results observed with glucose. we could not demonstrate elevated anaerobic growth on xylose. This was true even after previous anaerobic growth on glucose or following the addition of glucose. We note that. even though genes from *P. stipitis* enable *S. cerevisiae* to convert xylose to ethanol. transformed *S. cerevisiae* will only do so under aerobic conditions (Hahn-Hägerdal et al. 1996). Moreover. native *S. cerevisiae* will produce ethanol from xylulose. but requires mitochondrial activity to metabolize this sugar (Maleszka and Schneider 1984). This observation plus the inability of *ScURA1*-transformed *P. stipitis* to grow anaerobically on xylose suggest that there is a fundamental difference between glucose and xylose metabolism that involves oxygen. Bruinenberg et al. (1984) pointed out that cofactor imbalance could block anaerobic xylose metabolism by preventing the conversion of xylose to xylulose, but because the aldose reductase of *P. stipitis* will accept either NADH or NADPH. it is capable of fermenting xylose anaerobically at a slow rate. Although *P. stipitis* will ferment xylose slowly under anaerobic conditions, researchers recognized early that aeration increases ethanol production from xylose in this organism. so oxygen might play an additional role(s) in sugar transport or redox balance during xylose metabolism (du Preez et al. 1989). *P. stipitis* may also use different electron-transport chains to recycle reducing equivalents in supporting cell growth on xylose and glucose. This hypothesis is based on a previous study that discovered the alternative respiratory pathway in *P. stipitis* (Jeppsson et al. 1995). When CBS 6054 was grown on xylose in the presence of the cytochrome pathway inhibitor, cyanide, a 0.45 g/g ethanol yield was obtained. However, when the alternative respiratory pathway was blocked with salicyl hydroxamate on xylose. *P. stipitis* produced only 0.35 g/g ethanol. In addition. the presence of salicyl hydroxamate did not inhibit the respiration of xylose-grown cells but it did produce 11% inhibition of glucose-grown cells. Therefore. separate roles for each of the electron-transport pathways are implicated in supporting cell growth on xylose. Further. the different growth responses with xylose and glucose might be related to the machinery involved in sugar transport. Xylose transport is rate-limiting in *P. stipitis* (Ligthelm et al. 1988a) and both its high- and low- affinity uptake are mediated by a proton symport (Killian and van Uden 1988). thus anaerobic xylose transport may be very slow. Further investigation of *P. stipitis* transformed with *ScURA1* should reveal additional requirements for anaerobic growth on xylose.

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