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Disruption of the Cytochrome *c* Gene in Xylose-utilizing Yeast *Pichia stipitis* Leads to Higher Ethanol Production

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The xylose-utilizing yeast, *Pichia stipitis,* has a complex respiratory system that contains cytochrome and non-cytochrome alternative electron transport chains in its mitochondria. To gain primary insights into the alternative respiratory pathway, a cytochrome *c* gene (*PsCYC1,* Accession No. AF030426) was cloned from wild-type *P. stipitis* CBS 6054 by cross-hybridization to *CYC1* from *Saccharomyces cerevisiae.* The 333 bp open reading frame of *PsCYC1* showed 74% and 69% identity to *ScCYC1* and *ScCYC7,* respectively, at the DNA level. Disruption of *PsCYC1* resulted in a mutant that uses the salicylhydroxamic acid (SHAM)-sensitive respiratory pathway for aerobic energy production. Cytochrome spectra revealed that cytochromes *c* and $a{\cdot}a_3$ both disappeared in the *cyc1*-Δ mutant, so no electron flow through the cytochrome *c* oxidase was possible. The *cyc1*-Δ mutant showed 50% lower growth rates than the parent when grown on fermentable sugars. The *cyc1*-Δ mutant was also found to be unable to grow on glycerol. Interestingly, the mutant produced 0·46 g/g ethanol from 8% xylose, which was 21% higher in yield than the parental strain (0.38 g/g). These results suggested that the alternative pathway might play an important role in supporting xylose conversion to ethanol under oxygen-limiting conditions. Copyright © 1999 John Wiley & Sons, Ltd.

KEY WORDS — *Pichia stipitis;* cytochrome *c* gene; SHAM-sensitive respiration; gene disruption; xylose conversion

INTRODUCTION

In *Pichia stipitis,* respiro-fermentative and oxidative metabolism coexist to support cell growth and the conversion of sugar to ethanol (Ligthelm *et al.,* 1988). Unlike the Crabtree-positive yeast, *Saccharomyces cerevisiae, P. stipitis* requires low-level oxygenation (1·5-1·75 mM/1/h) to attain maximal rates of ethanol production (Delgenes *et al.,* 1986; Du Preez *et al.,* 1989; Skoog and Han-Hägerdal, 1990). In *P. stipitis,* oxygen limitation, rather than the increase of metabolites in the lower part of glycolysis, induces the fermentative alcohol dehydrogenase and pyruvate decarboxylase (Bruinenberg *et al.,* 1984; Du Preez *et al.,* 1989). However, ethanol is not formed in the presence of excessive amounts of oxygen (Du Preez *et al.,* 1994; Ligthelm *et al.,* 1988; Skoog and Han-Hägerdal, 1990). As oxygen becomes limiting, alcohol dehydrogenase (*ADH1*) and pyruvate decarboxylase (*PDC1*) increase 10-fold at the protein levels (Passoth *et al.,* 1996; Passoth *et al.,* 1998; Cho and Jeffries, 1999). Moreover, respiratory activity in *S. cerevisiae* is repressed by glucose under aerobic conditions, but in *P. stipitis,* respiration is not repressed by the presence of

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fermentable sugars or under oxygen-limited conditions (Passoth *et al.,* 1996). Following a shift from fully aerobic to oxygen-limited conditions, the total respiratory capacity in *P. stipitis* is not reduced, and the activity of a key respiratory enzyme, pyruvate dehydrogenase, is unchanged in xylose- or glucose-grown ceils.

In 1990, Alexander and Jeffries pointed out that some Crabtree-negative yeasts have alternative respiratory pathways in addition to the cytochrome pathway. Jeppsson *et al.* (1995) reported that *P. stipitis* has an alternative respiratory pathway that is resistant to cyanide or antimycin A but sensitive to salicylhydroxamic acid (SHAM). This SHAM-sensitive respiratory pathway is commonly present in fungi (Li *et al.,* 1996; Yukioka *et al.,* 1998), higher plants (Vanlerberghe and McIntosh, 1997) and a few yeast species, such as *Hansenula anomala* (Viola *et al.,* 1986) and *Schwanniomyces castellii* (Poinsot *et al.,* 1986). It branches off from the cytochrome pathway at the level of ubiquinone and donates electrons directly to oxygen to form water (Jeppsson *et al.,* 1995). Because the alternative oxidase is not a proton pump and two of the three sites for proton translocation are bypassed, only lower energy can be produced in this pathway via the linkage to the NADH dehydrogenase complex (Vanlerberghe and McIntosh, 1997). However, the physiological role and the composition of the SHAM-sensitive pathway remain unknown in *P. stipitis.*

In order to study the roles of alternative respiration in supporting cell growth and xylose conversion in *P. stipitis,* one needs to create mutants containing a crippled cytochrome pathway. To do so, a functional component of the cytochrome electron transport system needs to be cloned and disrupted. Cytochrome *c* is a good candidate because it is highly conserved among eukaryotes. More than 95 eukaryotic cytochrome *c* proteins have been studied to date. All of them share extensive homology, which is indicative of the very ancient origin and conserved function of the cytochrome respiratory system (Moore and Pettigrew, 1990). Cytochrome *c* is a small soluble heme protein that accepts electrons from the cytochrome bc_1 complex and donates them to the cytochrome *c* oxidase complex. By cloning and disrupting the cytochrome *c* gene in *P. stipitis* (*PsCYC1*), we could block the electron flow through the cytochrome *c* oxidase and force the cells to depend on the alternative respiratory pathway to generate energy. Therefore, we could assess the roles of the SHAM-sensitive pathway in supporting cell growth and xylose conversion when it acts as the sole energy-producing system under oxygen-limited or fully aerobic conditions. Our results will provide new insights into the SHAM-sensitive respiration system in a Crabtree-negative yeast and broaden our current knowledge on eukaryotic respiratory systems. Moreover, a better understanding of the respiratory machinery in *P. stipitis* will assist the development of this yeast as an industrial organism to produce ethanol from agricultural and pulping wastes.

MATERIALS AND METHODS

Microbial strains

The *P. stipitis* strains used in this study were wild-type CBS 6054 (NRRL Y-1145, ATCC 58785) and FPL-UC7 (*ura3-3,* NRRL Y-21448). *Escherichia coli* DH5a [F^- *recA1 endA1 hsdR17*(r_K^-, m_K^+) *supE44 thi-1 gyrA relA1*] (Gibco BRL) and XL-l Blue MRF′ (*recA*$^-$ *mcrA*$^-$ *mcrB*$^-$ *mrr*$^-$) were used for routine recombinant DNA experiments. XL-1 Blue and SOLR (Stratagene) strains were also used in conjunction with the *P. stipitis* l-ZAPII recombinant phage library.

Media and growth conditions

Yeast nitrogen base (YNB; Difco) without amino acids (1.7 g/l), 5 g/l ammonium sulphate, and 20 g/l glucose were used for yeast cultivation and transformation. Uridine was supplied at 20 mg/l for the growth of FPL-UC7. Yeast strains were cultivated at 30°C with shaking at 100 rpm for liquid cultures. *E. coli* was routinely cultivated at 37°C in Luria–Bertani medium supplemented with 50 µg/ml ampicillin when required.

Enzymes and primers

Restriction enzymes and other DNA modification enzymes were obtained from New England Biolabs, Stratagene, Promega, and Boehringer-Mannheim. Reaction conditions were as recommended by the suppliers. Primers were synthesized by Ransom Hill and Genosys.

Southern blot and library screening

Southern transfer by capillary blotting was performed according to Sambrook *et al.* (1989). DNA hybridization was conducted using a Genius™ 1

kit (Boehringer–Mannheim) with Nytran filters (Schleicher & Schuell). Hybridizations were performed in 25% formamide at 37°C. A *P. stipitis* λ-ZAPII recombinant phage library (Lu *et al.*, 1998a) was screened by using a *ScCYC1* probe labelled with digoxigenin. The *ScCYC1* fragment was from plasmid pAB458 (Fetrow *et al.*, 1989). Positive plaques from the initial screen were subject to two more rounds of screening. PBluescript II SK(–) was used as the vector to harbour the DNA from the positive clones.

DNA isolation and sequencing

Yeast genomic DNA was isolated by the method described in Rose *et al.* (1990). *E. coli* DNA isolation was carried out with a spin-Miniprep kit (Qiagen). 5 kb of the 5′ flanking region in Clone 1 was removed and the plasmid was religated to make pA234. *PsCYC1* in plasmid pA234 was sequenced by the University of Wisconsin Biotechnology Center, using a 370/3 ABI automated sequencer (Perkin Elmer).

Sequence alignment and taxonomic analysis

DNA sequence assembly, alignment and analysis were conducted by using the Genetics Computer Group sequence analysis software package (Devereux *et al.*, 1984). BLAST searches were performed on the National Center for Biotechnology Information server. The GCG package was used with its default setting for PILEUP analysis. Distances were calculated as substitutions per 100 amino acids, using the Kimura method (Kimura, 1983) following deletion of gapped regions. The phylogenetic tree was drawn using the neighbour-joining method.

Construction of a PsCYC1 *disruption cassette*

A one-step gene replacement method was employed to disrupt the *PsCYC1* gene. A 1·5 kb *PsURA3* fragment was excised as a *Bam*HI-*Xba*I fragment from pVY2 (Yang *et al.*, 1994) and subcloned into the *Bam*HI/*Xba*I sites in pUC19 to create pNQ21. A 644 bp fragment containing 588 bp of 5′ untranslated region (5′ UTR) of *PsCYC1* and the first 56 bp of the coding region was amplified with P1, 5′-CCGGGATCCATCAA CTCATCGACCTC-3′, and P2, 5′-CCGGGATC CGTCTTGAACAAGGTGGC-3′, containing a *Bam*HI site at the end of each primer. P1 annealed at – 588 to – 568 of the 5′UTR, while P2 annealed at +38 to +56 of the *PsCYC1* coding region. The PCR conditions were described in Shi and Jeffries (1998). The fragment was digested with *Bam*HI and cloned into the *Bam*HI site as pNQ22. Meanwhile, a fragment containing a truncated 83 bp *PsCYC1* coding region (+250 to +333) plus a 278 bp 3′ untranslated region (3′ UTR) was first cut out from pA234 and subcloned into the *Kpn*I/*Pst*I sites of pUC19 as pNQ13. Later, the same fragment was excised as a *Hin*dIII-*Eco*RI fragment and subcloned into the pBluescript II KS(+) to create pNQ23. This subcloning was to obtain the appropriate sites at each end. The same fragment was then excised as a *Pst*I–*Pst*I fragment from pNQ23 and subcloned into the *Pst*I site of pNQ22 after the *PsURA3* gene as pNQ26. The disruption cassette was liberated from pNQ26 by *Sma*I and *Sph*I and used to transform FPL-UC7.

Genetic transformation

E. coli transformation was performed as described in Sambrook *et al.* (1989). Yeast transformation was carried out by the lithium acetate method, as described in Rose *et al.* (1990). Putative transformants with small colony sizes were first picked on YNB-glucose (2%) minimal medium.

Screening of putative disruptants and confirmation

Thirty-three putative transformants were grown in 5 ml of YNB-glucose liquid medium at 30°C for 3-4 days. Genomic DNA was isolated and used as template for PCR screening. Two primers were used to screen the putative homozygous disruptants, P1 and P3: 5′-CTTACTTGGTGGCGG AAGCC-3′. P1 was one of the PCR primers described above, while P3 annealed at +314 to +334 of *PsCYC1*. To confirm the true homozygous disruptant strain, genomic DNA from FPL-Shi21 or FPL-UC7 or CBS 6054 was isolated, and digested by *Cla*I, *Bgl*II and *Sph*I, respectively. 20 μg of DNA was separated on a 0.8% TBE gel and then transferred to a Nylon filter. A *Hin*dIII-*Hin*dIII fragment of the 5′ UTR of *PsCYC1* was excised from pA234. This fragment was used as a probe to hybridize the filter. For the respiratory inhibitor experiment, YNB-glucose (2%) or YNB-xylose (2%) medium was prepared in the plates with four separate quarters. The concentration of antimycin A used was 5 μM while the concentration of SHAM was 4 mM.

Cytochrome spectra determination

Low temperature (– 196°C) spectrophotometric recordings of the FPL-UC7 and FPL-Shi21 strains were performed with whole cells. The strains were grown on 1% yeast extract, 2% peptone and 1% sucrose at 30°C for 3 days. The absorption spectra were recorded as previously described (Hickey *et al.,* 1991).

Aerobic growth experiments

Strains grown on YNB minimal medium containing either 2% glucose or xylose at 30°C for 4 days were used to inoculate 25 ml liquid cultures in 125 ml baffled flasks. Two replicate flasks of cultures were grown at 30°C with shaking at 160 rpm. The starting cell density was approximately 0·02 g/l (dry weight). The growth conditions described here were initially fully aerobic because no ethanol was produced (Passoth *et al.,* 1996; Cho and Jeffries, 1999). Samples were withdrawn regularly and the growth rate was measured by monitoring at OD_{600}. The growth experiment on non-fermentable carbon sources followed the same set-up and growth conditions except using 2% glycerol or 2% ethanol as the carbon source. The starting cell density was approximately 0·4 g/l (dry weight).

Xylose fermentation trial

Strains of FPL-Shi21, FPL-UC7 and CBS 6054 were grown for 3 days on fermentation medium (YNB 1·7 g/l, urea 2·27 g/l, peptone 6·56 g/l and xylose 80 g/l. Cells were harvested and used to inoculate 50 ml liquid medium in a 125 mL-Erlenmeyer flask. The starting cell density was approximately 1·9 g/l. The cultures were then grown at 25°C with shaking at 100 rpm. This is the standard growth condition we have developed for ethanol production during respiro-fermentative growth in xylose-utilizing yeasts (Jeffiies, 1982; Sreenath *et al.,* 1986; Cho and Jeffries, 1999). Samples from three replicate flasks were withdrawn daily to monitor the cell growth at OD_{600}. Cell yields were converted to g/l by dry weight determination from three replications on each strain. One OD_{600} unit of CBS 6054, FPL-UC7 or FPL-Shi21 was converted to 0·22, 0·21 and 0·20 g/l cells in dry weight, respectively. The fermentation sample tube was then spun at 14 000 rpm for 5 min and the supernatant was used for HPLC or GC analysis (Shi and Jeffries, 1998).

RESULTS AND DISCUSSION

Cloning and sequence analysis of the PsCYC1 *gene*

In order to generate the *cyc1*-Δ mutant, we cloned the cytochrome *c* gene from *P. stipitis.* To do so, a *ScCYC1* probe was used to screen 200 000 plaques from the *P. stipitis* λ–ZAPII recombinant phage library, from which three positive clones were identified. Only a single band was observed from these three overlapping clones in the Southern hybridizations and in the later restriction mapping analysis.

Sequence analysis revealed a 333 bp open reading frame that had only one potential TATA box located from –93 to –87 bp. However, the typical eukaryotic polyadenylation signal, AATAAAA, is not present within the 3′ UTR of *PsCYC1.* Instead, a sequence, TAG-N43-TAGT-N12-TTT. which is found 13 bp after the stop codon, might act as a putative transcription termination signal (Zaret and Sherman, 1982).

A BLAST search indicated that the cytochrome *c* of *P. stipitis* exhibited high sequence homology at the amino acid level to 14 other yeast and filamentous fungal cytochrome *c* proteins (Janbon *et al.,* 1997) except four regions with unique amino acids (data not shown). A taxonomic analysis was also performed on 10 yeast cytochrome *c* genes that showed close similarities. *Schizosaccharomyces pombe* was removed from the final yeast group because of its remote relationship with the nine other yeast *CYC* genes. *PsCYC1* displayed 74% and 69% sequence homology to *ScCYC1* and *ScCYC7* at the DNA level, respectively. As shown in the phylogenetic tree (Figure 1), the closest neighbour for the *PsCYC1* is the *CYC* gene from the starch-fermenting yeast, *Schwanniomyces occidentalis* (Amegadzie *et al.,* 1990). Interestingly, SHAM-sensitive respiration has also been reported from *Schwanniomyces castellii* (Poinsot *et al.,* 1986).

We also searched for the putative regulatory elements at the 5′ UTR of *PsCYC1.* A putative Hap1p binding site lying between – 126 and – 105 bp perfectly matched the *ScHAP1* consensus binding sequence (Ha *et al.,* 1996). In addition, two putative binding sites for the Hap2/3/4/5p complex were found, extending from – 152 to – 144 and – 136 to – 128 fit to the consensus (Guarente *et al.,* 1984; Forsburg and Guarente, 1988). These elements are clustered in a 48 bp region which is unique. The presence of the

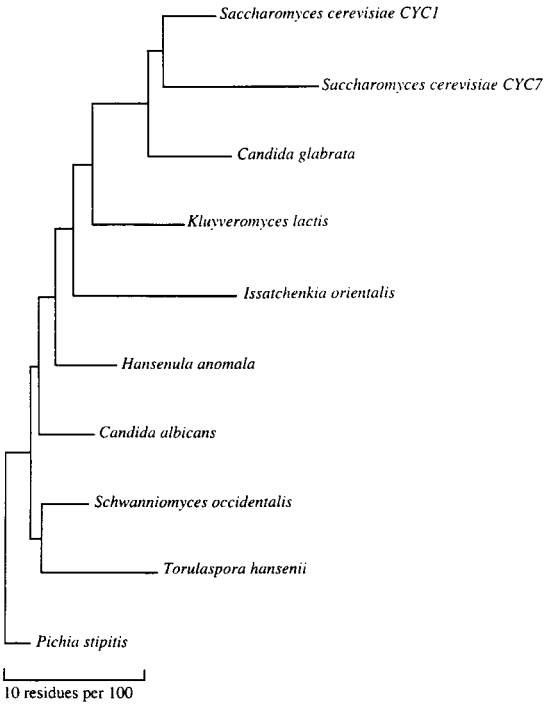

Figure 1. Phylogenetic relationship of *PsCYC1* to other *CYC* genes identified from nine different yeast species. Evolutionary distances were calculated from amino acid differences using the Kimura (1983) method, and the tree was drawn using the nearest neighbour-joining method.

putative Hap1p and Hap2/3/4/5p binding sites implied that the *PsCYC1* expression might be regulated by oxygen with involvement of heme. Results from our future experiments will shed some light on the regulatory cascade involved in oxygen-sensing in *P. stipitis.*

Disruption of the CYC1 *gene in* P. stipitis

Disrupting the *CYC1* gene in *P. stipitis* had two purposes. First, we needed to confirm that we had cloned the right gene. Second, we hoped to obtain a respiratory mutant in which the electron flux through the cytochrome *c* oxidase was blocked. To do that, a one-step gene replacement was employed, using the *PsURA3* gene to disrupt the *PsCYC1* coding region in a diploid strain, FPL-UC7 (Lu *et al.,* 1998b). A 1·5 kb *PsURA3* gene was fused to a 588 bp 5′ UTR plus the first 56 bp of the *PsCYC1* coding region. A 278 bp 3′ UTR plus an 83 bp coding region of *PsCYC1* was then ligated to the 3′-end of the *PsURA3* gene to make the disruption cassette, pNQ26 (Figure 2A).

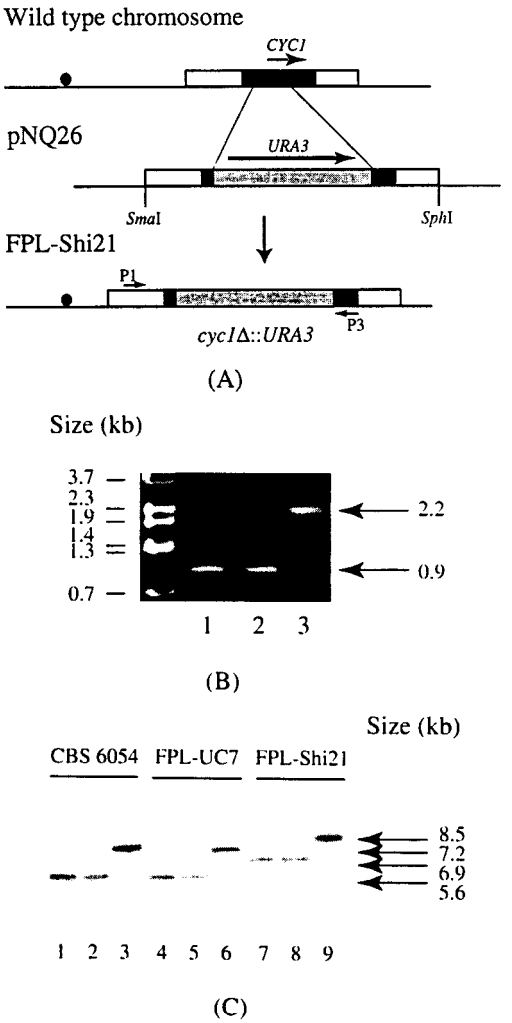

Figure 2. Disruption of the cytochrome ***c*** gene in *P. stipitis.* (A) Outline of the one-step gene replacement employed in disrupting *PsCYC1* to create FPL-Shi21. White boxes indicate the 5′ UTR and 3′ UTR of *PsCYC1.* The black box indicates the coding region of *PsCYC1.* The gray box indicates the functional *PsURA3* gene. The remaining *PsCYC1* coding region in pNQ26 is 139 bp. (B) PCR banding patterns from wild-type CBS 6054 (lane 1), FPL-UC7 (lane 2) and FPL-Shi21 (lane 3), using primers P1 and P3, shown in (A). Arrows indicate sizes of PCR products. (C) Results from the genomic Southern analysis on FPL–Shi21 and the control strains. Lanes 1, 4 and 7 are DNA-digested by *Cla*I. Lanes 2, 5, and 8 are DNA-digested by *Bgl* II. Lanes 3, 6, and 9 are DNA-digested by *Sph*I. Arrows indicate sizes of bands that hybridized to the *Hin*dIII-*Hin*dIII fragment of the 5′ UTR of *PsCYC1,* excised from pA234.

After transformation, 33 putative disruptants exhibiting smaller colony sizes were picked from YNB–glucose selective medium. They were then

screened by PCR, using primers P1 and P3. One strain, named FPL–Shi21 (NRRL Y-21971), showed a single 2·2 kb band corresponding to the total size of the 5′ UTR of *PsCYC1*, the *PsURA3*, and the remaining coding region of *PsCYC1* (Figure 2B). This strain was identified as a homozygous *cyc1*-Δ strain. The parental strain, FPL–UC7, showed a single 0·9 kb band corresponding to the size of the 5′ UTR plus the *PsCYC1* coding sequence. FPL–Shi21 was further confirmed by a genomic Southern blot (Figure 2C), using three different restriction enzymes that did not cut within the *PsCYC1*. The blot was probed with a *Hin*dIII-*Hin*dIII fragment of the 5′ UTR of *PsCYC1* excised from pA234. A single band of 6·9 kb, which was 1·3 kb bigger than the *PsCYC1* bands from the control strains, was observed from the *Cla*I or *Bgl*II digestion in FPL–Shi21. An 8·5 kb band was observed from FPL-Shi21 instead of a 7·2 kb band from the FPL–UC7 or CBS 6054 in the *Sph*I digestion. These results suggest that FPL–Shi21 is a homozygous disruptant. We also observed that the integration frequency of pNQ26 into the *P. stipitis* genome was low. This finding is in agreement with a previous report on the disruption studies of the *PsADH* genes (Cho and Jeffries, 1998). Heterozygous disruptants were observed in the screening, supporting the notion that the parental strain, FPL–UC7, is a diploid (Lu et *al.*, 1998b).

No electron flow goes through the cytochrome c *oxidase in FPL–Shi21*

Mutating cytochrome *c* in yeast and fungi usually affects the presence of other cytochrome species (Dumont *et al.*, 1987; Drygas *et al.*, 1989). To investigate whether disrupting *PsCYC1* could lead to changes in other cytochromes, we studied the cytochrome spectra of the *cyc1*-Δ mutant. In a preliminary experiment, FPL–UC7 was found to contain normal levels of cytochromes *c*, c_1, *b*, and $a{\cdot}a_3$. The heights and the positions of the peaks were the same as observed from the wild-type CBS 6054 (data not shown). Low temperature (− 196°C) spectrophotometric recordings were subsequently performed on whole cells of FPL–Shi21 and FPL–UC7 grown on 1% sucrose medium. The peaks of cytochromes $a{\cdot}a_3$, *b*, c_1 and *c* are located, respectively, at 602·5, 558·5, 553·3 and 547·3 nm (Figure 3). In contrast, FPL–Shi21 is completely deficient in cytochromes *c* and $a{\cdot}a_3$, partially deficient in cytochrome c_1 and has an increased level of cytochrome *b*. The cytochrome

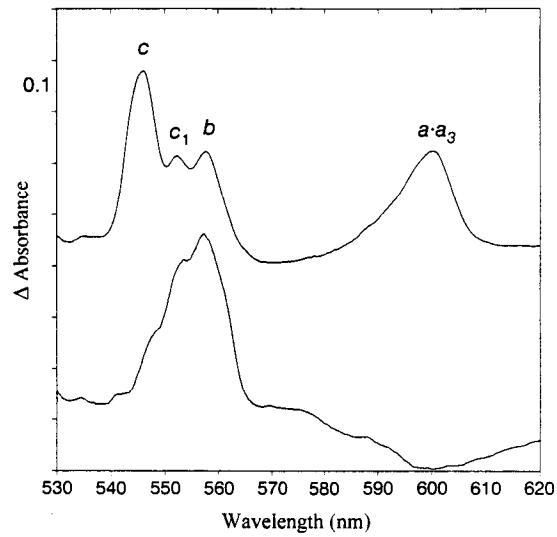

Figure 3. Low temperature (− 196°C) spectrophotometric recordings on whole cells of FPL–Shi21 (bottom curve) and the parental strain, FPL–UC7 (top curve), grown on 1% sucrose medium.

spectrum pattern of FPL–Shi21 resembles mutants of *Saccharomyces cerevisiae* that lack cytochrome *c* (Downie *et al.*, 1977). It has been reported that in *S. cerevisiae*, mutants lacking cytochrome *c* are also deficient in cytochrome $a{\cdot}a_3$ due to a secondary effect of the cytochrome *c* deficiency (Sherman *et al.*, 1965; Downie *et al.*, 1977; Dumont *et al.*, 1987). The lack or diminished level of cytochrome $a{\cdot}a_3$ has also been observed in mutants of *Neurospora crassa* deficient in cytochrome *c* (Bottorff *et al.*, 1994; Drygas *et al.*, 1989; Nargang *et al.*, 1988). The co-disappearance of $a{\cdot}a_3$ suggested that no electron flow could go through the cytochrome *c* oxidase in the *cyc1*-Δ mutant of *P. stipitis*. These results indicated that we had obtained an ideal mutant that would allow us to study the SHAM-sensitive pathway in *P. stipitis*.

We also performed a respiratory inhibitor study to confirm that the SHAM-sensitive pathway was the only respiratory pathway for aerobic energy production in FPL–Shi21. Antimycin A, which blocks electron transfer from the cytochrome bc_1 complex to cytochrome *c* and SHAM, which blocks the electron transfer to the alternative oxidase (Figure 4A), were used in the study. Three-day-old cells of FPL–Shi21 and FPL–UC7 were plated on YNB minimal medium containing either 2% glucose or xylose supplemented with 5 μM antimycin A alone, 4 mM SHAM alone, or both

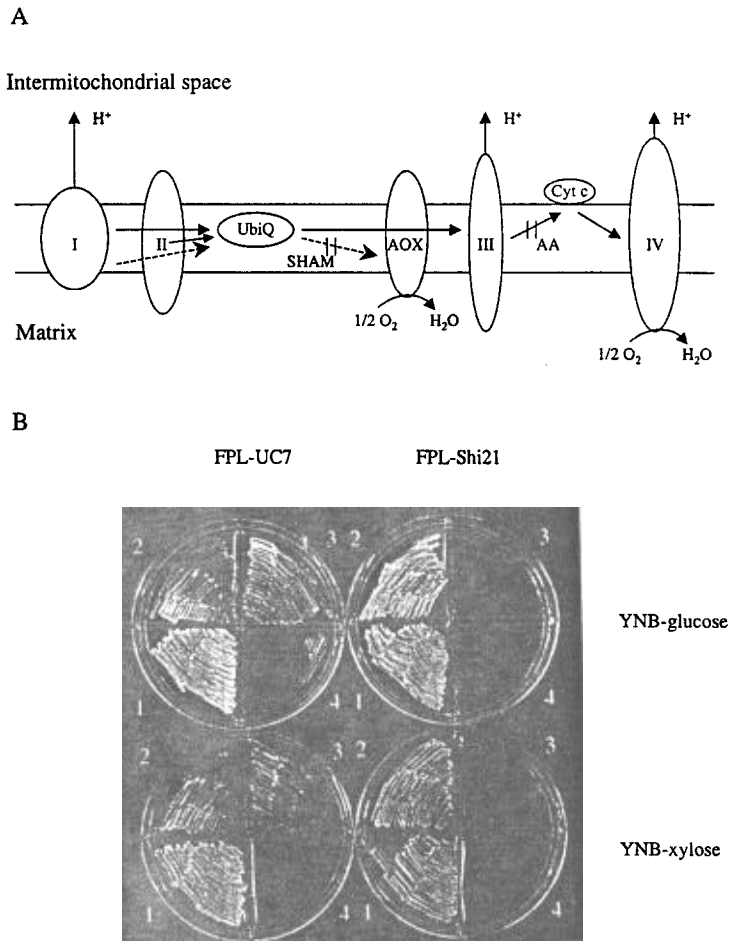

Figure 4. (A) Schematic diagram of the electron transport systems in *P. stipitis*. The solid lines with arrows indicate the cytochrome pathway, while the dotted lines with arrows indicate the SHAM-sensitive alternative pathway. The action points of antimycin A and SHAM are labelled: I, NADH dehydrogenase complex; II, succinate dehydrogenase complex; UbiQ, ubiquinone complex; AOX, alternative oxidase; III, cytochrome bc_1 complex; Cyt c, cytochrome *c*; IV, cytochrome *c* oxidase; AA, antimycin A; SHAM, salicylhydroxamic acid. (B) Results from the respiratory inhibitor study in cells of FPL–UC7 and FPL–Shi21 grown on YNB–glucose (2%) or YNB–xylose medium (2%). Sector 1, no respiratory inhibitors added; Sector 2, antimycin A (5 μM); Sector 3, SHAM (4 mM); Sector 4, antimycin A (5 μM) and SHAM (4 mM).

respiratory inhibitors (Figure 4B). Appropriate concentrations of the inhibitors were determined from preliminary experiments. Cells of FPL-Shi21 could not grow on either xylose or glucose medium when SHAM was present alone (sector 3 of FPL-Shi21). Cells of FPL-Shi21 also showed insensitivity to antimycin A when it was present alone (sector 2 of FPL-Shi21). In contrast, the parental strain, FPL-UC7, could use either the cytochrome or the SHAM-sensitive respiratory pathway to support growth (sectors 2 and 3 of FPL–UC7). These results demonstrated that the SHAM-sensitive pathway was the only functional respiratory pathway in FPL–Shi21.

Initial characterization of the cyc1-Δ mutant

To assess the physiological roles of the alternative respiratory pathway, we tested the growth

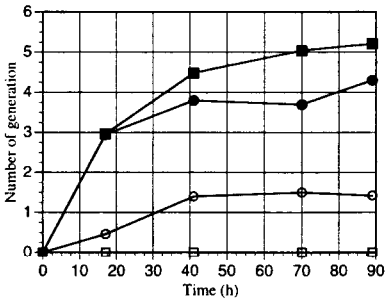

Figure 5. Results of the aerobic growth experiment of FPL–Shi21 and FPL–UC7 on non-fermentative carbon sources. (■) FPL–UC7 grown on YNB–glycerol (2%) medium, (●) FPL–UC7 grown on YNB–ethanol (2%) medium, (□) FPL–Shi21 grown on YNB–glycerol (2%) medium, and (○) FPL–Shi21 grown on YNB-ethanol (2%) medium. Uridine (20mg/l) was added for the growth of FPL–UC7.

abilities of the *cyc1*-Δ mutant on various carbon sources. Under fully aerobic conditions, the growth rate of the *cyc1*-Δ mutant was about 50% of the parent when cultivating on glucose or xylose (data not shown). The lower cell mass produced by the *cyc1*-Δ strain indicated that the SHAM-sensitive pathway could produce some energy to support growth. This energy probably results from the linkage to proton translocation at NADH dehydrogenase complex (see Figure 4A). We then tested the growth ability of the *cyc1*-Δ mutant on non-fermentable carbon sources. The *cyc1*-Δ mutant was unable to grow on glycerol under fully aerobic conditions (Figure 5) which indicated that the SHAM-sensitive pathway could not support respirative growth on glycerol. However, this mutant grew 1·5 generations on ethanol, which was not expected. This could be due to the reassimilation of ethanol in *P. stipitis* via a unique ADH system with fermentative and respirative functions (Cho and Jeffries, 1998; Passoth *et al.*, 1998). The same fermentative ADH1 can convert ethanol to acetaldehyde, then to acetate. However, the accumulation of acetate can collapse the membrane motive force in *P. stipitis* (Meyrial et al., 1997), therefore only some growth was permitted.

The *cycl*-Δ strain was also tested for its respiro-fermentative capacity on xylose by comparison with the parental strains, FPL–UC7 and CBS 6054 (Table 1). The cell yield from the mutant strain grown under oxygen-limited conditions was one-half that of the parental strains. On the other hand, the mutant strain appeared to use xylose slightly faster than the control strains. Moreover, the ethanol production rate from the mutant on xylose was faster than that from the control strains. No significant amounts of xylitol or other polyols were produced from the mutant. Notably, the ethanol yield was 21% higher than its parent, FPL–UC7 and 35% higher than the wild-type CBS 6054 under the conditions tested. These results suggested that downregulating the cytochrome respiration could lead to higher ethanol production efficiently in *P. stipitis.* Similar observations have been reported by Hutter and Oliver (1998) from two nuclear petite mutants bearing mutations in the cytochrome *c* oxidase in *S. cerevisiae.* Our data also demonstrated that the alternative respiratory pathway alone was able to support cell growth and xylose conversion to ethanol. The behaviour of the *cyc1*-Δ mutant in *P. stipitis* resembles to a cytochrome *b* mutant of *Schwanniomyces castellii* that uses the alternative respiration system to support glucose conversion to ethanol (Zimmer *et al.*, 1997). Further characterizations are under way to address other properties of the mutant and the regulation of the alternative respiration at the molecular *level* in *P. stipitis.*

Table 1. Fermentation trial on 8% xylose medium by different strains of *P. stipitis.*

Fermentation parameters	CBS 6054 (wild-type)	FPL–UC7 (*ura3*)	FPL–Shi21 (*cyc1*-Δ)
Biomass yield ($Y_{X/S}$)[a]	0·16	0·17	0·09
Ethanol yield (YP/S)[b]	0·34	0·38	0·46
Specific ethanol production rate (Q_P)[c]	0·04	0·03	0·06
Specific xylose uptake rate (Q_S)[d]	0·11	0·09	0·13

[a] $Y_{X/S}$ (g (dry weight) . g xylose $^{-1}$).
[b] $Y_{P/S}$ (g ethanol . g xylose $^{-1}$).
[c] Q_E (g ethanol . g (dry weight)$^{-1}$. h $^{-1}$).
[d] Q_s (g xylose . g (dry weight)$^{-1}$. h $^{-1}$).

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