Yeast
Yeast 2002; **19:** 1203–1220.
Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/yea.915

Research Article

SHAM-sensitive alternative respiration in the xylose-metabolizing yeast *Pichia stipitis*

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Received: 20 December 2001
Accepted: 28 July 2002

Abstract

SHAM-sensitive (STO) alternative respiration is present in the xylose-metabolizing, Crabtree-negative yeast, *Pichia stipitis,* but its pathway components and physiological roles during xylose metabolism are poorly understood. We cloned *PsSTO1,* which encodes the SHAM-sensitive terminal oxidase (PsSto1p), by genome walking from wild-type CBS 6054 and subsequently deleted *PsSTO1* by targeted gene disruption. The resulting *sto1*-D deletion mutant, FPL-Shi31, did not contain other isoforms of Sto protein that were detectable by Western blot analysis using an alternative oxidase monoclonal antibody raised against the Sto protein from *Sauromatum guttatum.* Levels of cytochromes *b, c,* c_1 and $a{\cdot}a_3$ did not change in the *sto1*-D mutant, which indicated that deleting PsSto1p did not alter the cytochrome pool. Interestingly, the *sto1*-D deletion mutant stopped growing earlier than the parent and produced 20% more ethanol from xylose. Heterologous expression of *PsSTO1* in *Saccharomyces cerevisiae* increased its total oxygen consumption rate and imparted cyanideresistant oxygen uptake but did not enable growth on ethanol, indicating that PsSto1p is not coupled to ATP synthesis. We present evidence that the mitochondrial NADH dehydrogenase complex (Complex I) was present in wild-type CBS 6054 but was bypassed in the cells during xylose metabolism. Unexpectedly, deleting PsSto1p led to the use of Complex I in the mutant cells when xylose was the carbon source. We propose that the non-proton-translocating NAD(P)H dehydrogenases are linked to PsSto1p in xylose-metabolizing cells and that this non-ATP-generating route serves a regulatory function in the complex redox network of *P. stipitis.* Published in 2002 by John Wiley & Sons, Ltd.

Keywords: ***Pichia stipitis*; Crabtree-negative yeast; SHAM-sensitive respiration; alternative oxidase; xylose conversion**

Introduction

Fungi and higher plants differ from other eukaryotes by possessing branched electron transport chains (ETC) that include alternative components in addition to the standard respiratory system found in animals (Joseph-Home *et al.,* 2001). These alternative components lie at the initial electron entry site (Site I) and the final quenching site (Site IV) of the ETC (Figure 1). Upstream of the ETC, rotenone-insensitive NAD(P)H dehydrogenases (non-proton-translocating) are present in addition to the rotenone-sensitive NADH dehydrogenase Complex I (proton-translocating) (Rasmusson *et al.,* 1998). Downstream, an alternative terminal oxidase that is sensitive to salicylhydroxamic acid (SHAM) is present in addition to the standard cytochrome *c* oxidase (Cox) (Vanlerberghe and McIntosh, 1997). This SHAM-sensitive terminal oxidase (Sto) has been widely reported from

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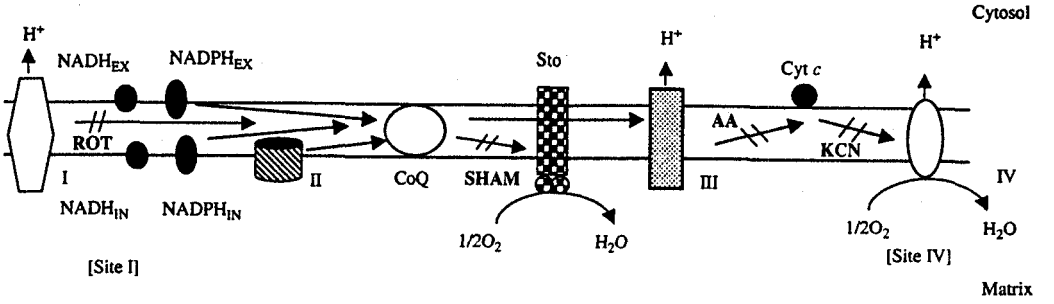

Figure 1. A diagram of alternative and standard redox components present in the electron transport chain (ETC) of Crabtree-negative yeasts, such as *Pichia stipitis.* The action site of SHAM, antimycin A (AA), rotenone (ROT) and KCN are marked. I, rotenone-sensitive NADH dehydrogenase complex (Complex I); $NADH_{IN}$, rotenone-insensitive NADH dehydrogenase (internal); $NADH_{EX}$, rotenone-insensitive NADH dehydrogenase (external); $NADPH_{IN}$, rotenone-insensitive NADPH dehydrogenase (Internal); $NADPH_{EX}$, rotenone-insensitive NADPH dehydrogenase (external); II, succinate dehydrogenase complex (Complex II); CoQ, ubiquinone complex, Sto, SHAM-sensitive terminal oxidase; III, cytochrome bc_1 complex: Cyt c, cytochrome c; IV, cytochrome c oxidase (Cox). Site I, electron entry site; Site IV, electron quenching site

yeasts (Viola *et al.,* 1986; Poinsot *et al.,* 1986), fungi (Lambowitz and Slayman, 1971; Downie and Gardland, 1973; Lloyd and Edwards, 1977) and higher plants (Douce and Neuburger, 1989).

Although the SHAM-sensitive respiration system (STO) was first discovered 70 years ago (Keilin, 1929), its functional components and physiological roles in yeasts and fungi remain unclear. Most of the current information on the biochemical and regulatory aspects of Sto proteins has been obtained from the studies of plant mitochondria. Structurally, the STO pathway branches from the cytochrome pathway (CYT) at the level of ubiquinone just before cytochrome *b* (Storey, 1976; Siedow, 1982). From this point, electrons are directly donated to Sto, which then reduces molecular oxygen to water. Sto itself is not coupled to ATP synthesis (Moore and Rich, 1985), so this alternative route bypasses at least two out of the three ATP-generating sites, and it is considered a pathway that conserves no energy by synthesis of ATP in plants.

Sto proteins from yeasts and fungi display significant differences in structure and regulation from their plant counterparts. Plant Sto proteins have di-iron centres in their active sites (Bonner *et al.,* 1986; Minagawa *et al.,* 1990; Moore *et al.,* 1995a; Moore *et al.,* 1995b). This separates them completely from the bacterial alternative terminal oxidases, which use heme in their active sites (Gennis and Stewart, 1996). At the transcriptional level, unlike the constant presence of low level of STO transcripts in plants, STO transcripts in yeasts and fungi only accumulate when the CYT system is suppressed (Yukioka *et al.,* 1998; Siedow and Umbach, 2000). Umbach and Siedow (2000) further demonstrated that certain fungal Sto proteins are monomeric, which differentiates them from the dimeric structure reported in plant Sto proteins. Thus, the fungal Sto proteins are normally present in a reduced (active) state while the plant Sto proteins need to be activated from an oxidized (less active) to its reduced state (active) (Umbach and Siedow, 1996; Rhoads et al., 1998). In addition, fungal Sto proteins lack certain conserved amino acids that are involved in mediating the activation process (Albury *et al.,* 1998; Rhoads *et al.,* 1998). These observations imply that the yeast and fungal Sto proteins may play other important physiological roles.

Recently, Veiga *et al.,* (2000) surveyed large numbers of yeast species in which STO respiration was detected. The list mainly encompasses yeasts that are unable to produce ethanol in the presence of fermentable sugars under strictly aerobic conditions (Crabtree-negative). *Pichia stipitis* has the characteristics of a Crabtree-negative yeast (Passoth *et al.,* 1996; Jeffries and Shi, 1999) and it possesses both CYT and STO respiration systems in its mitochondria (Jeppsson *et al.,* 1995; Shi *et al.,* 1999; Shi, 2000). Oxygen limitation, rather than the increase of metabolites in the lower part of

glycolysis, induces the onset of ethanol formation in *P. stipitis* (Bruinenberg *et al.*, 1984; Alexander and Jeffries, 1990; Cho and Jeffries, 1999). In addition, *P. stipitis* requires low-level oxygenation to attain maximal rates of ethanol production (Delgenes *et al.*, 1986: Du Preez *et al.*, 1989; Skoog and Hahn-Hägerdal, 1990).

Our previous study on a *cyc1*-D mutant of *P. stipitis* provided initial insight into the role of the STO system (Shi *et al.*, 1999). The *cyc1*-D mutant, which contains a defective CYT pathway, could survive and sustain growth on xylose or glucose — albeit at a significantly lower level — via the connection to Complex I, which is coupled to ATP synthesis. We also noted that the xylose-grown cells of the *cyc1*-D mutant showed increased total oxygen uptake rates to compensate the loss of the CYT pathway. Therefore, we could not completely rule out the possibility that PsSto1p itself is proton-motive. Moreover, the physiological role of the STO system during xylose metabolism in *P. stipitis* remained to be elucidated. To further understand the functions of the STO system and dissect the respiration network in *P. stipitis*, we cloned the *PsSTO1* gene from wild-type CBS 6054 and created a mutant in which *PsSTO1* is deleted. In the studies reported here, we characterized the *sto1*-D mutant of *P. stipitis.* and expressed the *PsSTO1* gene in a Crabtree-positive yeast, *Saccharomyces cerevisiae,* which does not have Complex I, independent NADPH dehydrogenases or Sto. The expression study in *S. cerevisiae* not only allowed us to determine whether PsSto1p is coupled to ATP synthesis but also to test the changed stoichiometry in its ETC after gaining an additional electron acceptor. We further extended our findings to the upstream of *P. stipitis* ETC to study the components present at Site I. This information is essential for us to understand the origin of the electron flow that is donated to PsSto1p. Our initial data indicate that *P. stipitis* has an intricate structure at Site I, which reflects the complexities of the respiratory networks present in Crabtree-negative yeasts and fungi.

Materials and methods

Microbial strains and maintenance

Microbial strains and plasmids used in this study are listed in Table 1. *P. stipitis* strains were cultivated at 30°C in YNB minimal medium containing 1.7 g/l yeast nitrogen base without amino acids (YNB; Difco), 5 g/l ammonium sulphate

Table 1. *P. stipitis* strains and plasmids used in this study

Strain or plasmid	Relevant genotype and characteristics	Reference or resource
P. stipitis		
CBS 6054	Wild-type (NRRL Y-1145)	Centraalbureau voor Schimmelcultures (Ultrecht, The Netherlands)
FPL-UC7	*ura3-3* (NRRL Y-21448)	Lu *et al.*, 199
FPL-Shi21	*cyc1::URA3* (NRRL-Y2197)	Shi *et al.*, I999
FPL-Shi31	*sto1::URA3* (NRRL-30230)	This work
S. cerevisiae		
679 a	*MAT* a *trp1-1*	Dr. Culbertson (University of Wisconsin. Madison, WI)
679 a (pNQ32)	A strain containing the *PsSTO1* expression Cassette. pNQ32	This work
679 a (pYPR2831)	A control strain containing the empty vector, pYPR2831	This work
Plasmids		
pNQ28	Contains the 293 bp centre region of *PsSTO1*	This work
pNQ30	Contains the 3 kb *PsSTO1* gene and its flanking regions	This work
pNQ31	Contains a disruption cassette for *PsSTO1*	This work
pNQ32	Contains the expression cassette of PsSTO1 in pYPR2831	This work
pYPR2831	Contains the *ScTDH1* promoter and terminator	Horiuchi *et al.*, 1990

and 20 g/l glucose. For yeast transformation, host strains were grown in YPD medium consisting of yeast extract, 10 g/l, peptone, 20 g/l. and glucose, 20 g/l. For general growth experiments, yeast strains were cultivated in medium containing 1.7 g/l yeast nitrogen base (YNB) without amino acids, 2.27 g/l urea, 6.56 g/l peptone (YNBUP), and 20 g/l sugar. *Escherichia coli* was cultivated at 37°C in Luria–Bertani medium supplemented with 100 µg/ml ampicillin or 50 µg/ml kanamycin when required.

Enzymes, primers and chemicals

Restriction enzymes, DNA modification enzymes and molecular reagents were obtained from New England Biolabs, Promega, Stratagene and Roche Biochemicals. Reaction conditions were as recommended by the suppliers. DNA preparations were performed using Qiaprep™ Spin Miniprep columns (Qiagen). Primers were synthesized by Sigma-Genosys and the sequences are listed in Table 2.

DNA sequencing

Sequences of all DNA clones were performed by using a ABI 371 automated DNA sequencer (Perkin-Elmer). DNA sequence analysis was conducted by using the Genetics Computer Group sequence analysis software package and DNA-MAN™ (Lynnon BioSoft). Phylogenetic studies were conducted using the neighbour-joining method (Kimura, 1983).

Genome walking to clone the *PsSTO1* gene

Two highly conserved regions of the known fungal alternative oxidases IFLES(I/V)AGVPGMV and HRFVGYLEEEAV were selected to design a pair of degenerate primers. Primers 1 and 2 were used to amplify a 293 bp region of *PsSTO1* from *P. stipitis* CBS 6054 (wild-type) with the *Pfu* DNA polymerase (Stratagene). The PCR reaction was carried out at 95°C for 5 min as the initial cycle, followed by 36 cycles of 95°C for 60 s, 55°C for 60 s and 72°C for 90 s. The final extension time was 15 min at 72°C. PCR products (1 µl) were directly cloned into the PCR2.1-TOPO vector (Invitrogen) to create pNQ28. The 293 bp fragment was sequenced and compared to the consensus of the fungal alternative oxidase genes. This region became the starting point for genome walking to clone the entire gene.

The Universal GenomeWalker™ kit (Clontech) was used to clone *PsSTO1*. Five individual linear DNA fragment libraries from wild-type CBS 6054 were constructed by using blunt-end restriction enzymes *Dra*I, *Eco*RV, *Pvu*II. *Stu*I and *Sca*I. The blunt-ended linear fragments were then ligated to the adapters provided by the manufacturer. These became the templates for genome walking PCR reactions. Gene-specific primers were designed based on the 293 bp region of *PsSTO1* for walking in each direction. The adapter primers (3 and 4) were supplied by the manufacturer. For the 5′ end walking reactions, primers 3 and 7 were used in the primary PCR, while primers 4 and 8 were used in the secondary PCR. For the 3′ end walking reactions, primers 3 and 5 were used in the primary

Table 2. DNA primers used in this study

Primer number	Primer sequence
1	5′-ATITTCCTYGAATCYRTYGCYGGIGTYCCWGG-3′
2	5′-ACRGCYTCYTCYTCIARGTADCCRACGAATCTGTG-3′
3	5′-GTAATACGACTATAGGGC-3′ (AP-1, Clonetech)
4	5′-ACTATAGGGCACGCGTGGT-3′ (AP-2, Clonetech)
5	5′-CCGTTGCTTCCTTCATCAGACACTTGCAT-3′
6	5′-GGTGTATTCTGCAACTTGTTCTTCTTGT-3′
7	5′-AAGAATGCAAGTGTCTGATGATGAAGGAAGCAA-3′
8	5′-GAACAAGAAGAACAAGTTGCAGAATACAC-3′
9	5′-GGCTCGTCTTTACGTCTTCGCATCTCAT-3′
10	5′-GCATGTGAAGACTTGAACGGGTTGACTT-3′
11	5′-ATGCTTCTGTGCAGACTACAAGACC-3′
12	5′-TTACAATTTCAATTCTTCCTTCCC-3′
13	5′-CCATCGATGGGAGCCGTTGTCTGAGAAG-3′
14	5′-CCATCGATGGAATAGGCCTCTGCTTGT-3′

PCR, while primers 4 and 6 were used in secondary PCR. The Advantage PCR™ kit (Clontech) was used in conjunction with the *Tth* DNA polymerase in all the PCR reactions for walking. The PCR conditions were as recommended by the manufacturer's manual. The secondary PCR products were directly cloned into the PCR2.1-TOPO vector for DNA sequencing. Based on the sequences, primers 9 and 10 were used to amplify a 3 kb *PsSTO1* fragment with the *Pfu* DNA polymerase (Stratagene). This fragment, which contained about l kb of each flanking region and the coding region, was cloned into the PCR–BluntII–TOPOvector (Invitrogen) as pNQ30. The GenBank Accession No. for *PsSTO1* is AY004212.

mRNA isolation and RT–PCR

Cells of CBS 6054 were pre-grown in YNB minimal medium containing 2% xylose for 2 days. The cells were then cultivated in 25 ml YNBUP liquid medium in a 125 ml baffled flask shaken at 160 rpm at 30°C (Shi *et al.,* 1999). Under this growth condition, no ethanol production is detected (Passoth et al., 1996; Cho and Jeffries, 1999). mRNA was extracted using a Dynabeads mRNA Direct™ kit (Dynal). 100 µl cells were mixed with 1 ml lysis buffer and Dynabead Oligo $(dT)_{25}$ for 5 min. The mRNA was separated by a Magnetic Separation Stand (Promega) and washed twice with the washing buffer. The mRNA was eluted at 65 °C and stored at –80°C for future use. Reverse transcription PCR (RT–PCR) was performed by using a Superscript One-Step RT–PCR System (Life Technologies). The reaction volume was 50 µl containing 0.3 µg mRNA, 0.2 µM each primer (11 and 12), 25 µl 2× reaction mix, 1 µl Superscript™ RT/Taq DNA polymerase mix. cDNA was synthesized at 50°C for 3 min and denatured at 94°C for 2 min. Then RT–PCR was carried out by running a program consisting of 40 cycles: 94°C for 15 s, 55 °C for 30 s and 72 °C for 80 s. The final RT–PCR reaction was terminated with 1 cycle of 72°C for 10 min. The primers for RT–PCR of *PsSTO1* were 11 and 12.

Construction of a *sto1*-D mutant

A one-step gene replacement method was employed to disrupt the *PsSTO1* gene in our parental strain, FPL-UC7 (Lu *et al.,* 1998). A 1.4 kb *PsURA3* fragment was amplified from pVY2 (Yang *et al.,* 1994) using *Pfu* DNA polymerase by primers 13 and 14, with a *Cla*I site at each end. The PCR conditions were the same as described in Shi and Jeffries (1998). This fragment was digested with *Cla*I, and sub-cloned into the *Cla*I site of the *PsSTO1* coding region in pNQ30 to create pNQ31.

Yeast transformation

General yeast transformation was carried our by using an EZ-Transformation kit (Biol01). To create the *sto1*-D mutant, the disruption cassette was liberated from pNQ31 by *Nde*I and *Eco*RI, and used to transform FPL-UC7. For *P. stipitis* transformation, the lithium acetate method (Ito *et al.,* 1983) in conjunction with restriction enzyme-mediated integration (REMI) was used to increase the transformation frequency (Sánchez *et al.,* 1998).

Screening of putative *sto1*-D mutants by colony PCR

Yeast cells (2 µl) were lysed using a Yeast Whole Cell Lysis kit (Biol01) with incubation at 37 °C for 1 h. The resulting lysate was mixed with 200 µM dNTPs, 10 µM each primer, 10× PCR buffer, 1.5 mM $MgCl_2$ to 99 µl volume, and heated at 95°C for 15 min. Five units of the *Taq* DNA polymerase (Roche) were added to the mixture, and the PCR reaction was run with 40 cycles of 95°C for 1 min, 50°C for 1 min and 72°C for 1 min. The PCR reaction was terminated with 4 min of final extension at 72°C. Primers 11 and 12 were used to screen the putative disruptants.

Mitochondria isolation and protein analysis

For mitochondrial protein analysis, *P. stipitis* cells were grown overnight in a medium containing 3 g/l yeast extract, 3 g/l malt extract and 5 g/l xylose. The yeast cells were grown in 25 ml medium in a 125 ml baffled flask shaken at 160 rpm at 30 °C (Govinden, 1995). Overnight-grown culture (15 ml) was used to inoculate a 2 1 flask containing 500 ml fresh medium. The culture was shaken at 160 rpm at 30°C for 24 h. Two flasks containing a total 1 I culture broth were harvested and the cells were treated with 0.5 mg/ml Novozyme 234 (Novozymes) in 5 ml solution A containing 0.5 M sorbitol, 10 mM Tris–HCl

(pH 7.5). Mitochondria were then separated from other organelles by sucrose gradient ultracentrifugation (Defontaine *et al.*, 1990; Querol and Barrio, 1991).

Mitochondrial proteins were released by using glass beads in a cell disruption buffer containing 100 mM sodium phosphate, 1 mM EDTA and 5 mM b-mercaptoethanol (Ciriacy, 1975). After vortexing three times for 1 min in a glass tube, the mixture was transferred to a new tube, which was then spun down at 16000 x *g* for 2 min and the top layer was transferred to a new tube. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay system (Pierce). Mitochondrial proteins (15 µg) were loaded on a 10–20% Tris–HCl reducing SDS–PAGE gradient gel (Bio-Rad). After electrophoresis for 40 min at 200 V, the protein was transferred to a nytran membrane (Schleicher & Schuell). The blot was hybridized with a 1 : 100 dilution of a mouse monoclonal antibody raised against the Sto protein of *Sauromatum guttatum* (Elthon *et al.*, 1989), then the blot was cross-reacted with an anti-mouse secondary antibody conjugated with an alkaline phosphatase at a 1 : 5000 dilution (Promega). The colour was developed by the NBT/BCIP system (Promega).

Oxygen uptake measurement

Oxygen uptake was measured by using a Clark electrode (Yellow Spring Instruments). The assay was performed in an assay buffer containing 20 mM potassium phosphate, 5 mM magnesium chloride, plus 20 g/l xylose or glucose as the substrate at 30 °C (Jeppsson *et al.*, 1995). The assay buffer was saturated with air before the experiment. Normally, 20–30 µl cells were added to the assay chamber and the total reaction volume was 2 ml. Oxygen uptake rates were calculated based on a dissolved oxygen concentration of 237 µM in air-saturated water at 30°C.

Potassium cyanide was dissolved in water, while antimycin A, SHAM or rotenone was dissolved in N,N-dimethyl-formamide. Up to 50 µl pure N,N-dimethyl-formamide does not affect respiration of yeast cells (Henry *et al.*, 1974). In our studies, the inhibitor stock solution was prepared at a concentration such that less than 50 µl was used in each measurement. The concentration of respiratory inhibitor was tested in previous trials. Inhibitor solution was injected to the chamber after adding the cells. In general, the cell volume used in each measurement was 10–30µl.

For the rotenone sensitivity experiment, cells of CBS 6054 were grown in 500 ml YNBUP medium using glucose or xylose as the carbon source in a 1 1 flask. The cells were handled as described in Jeppsson *et al.* (1995) and above. Two replicates were run in each measurement. The assay substrate for xylose-grown cells was 5 mM xylose, while the substrate for glucose-grown cells was 5 mM glucose; 30 µl cells were used in each measurement. For xylose- or glucose-grown cells, the cell amount (dry weight) used in each measurement was 1.4 mg.

Dry weight determination and HPLC analysis

Cells were spun down at 16 000 x *g* for 5 min, washed with sterile water for three times, then desiccated in a 105°C oven overnight. At least three replicates of samples were taken. Optical densities were determined at the same time. A 1.0 OD_{600} cell suspension of FPL-UC7 or FPL-Shi31 was equivalent to 0.22 g dry weight of cells/l, while for FPL-Shi21, 1.0 OD_{600} was equivalent to 0.20 g/l. For analysing fermentation end products, samples were drawn daily and spun at 16 000 x *g* for 5 min. The supernatant solution was diluted and subjected to high performance liquid chromatography (HPLC) analysis by using an ICSep-ION-300 column (CETAC Technologies). The column was operated at 60 °C with a 0.4 ml/min flow rate, using 0.005 M sulphuric acid as the solvent. Glucose, xylose, xylitol and ethanol are eluted at 16.3 min, 17.5 min, 22.8 min and 34.8 min, respectively.

Shake-flask fermentation

Cells of FPL-Shi31 and FPL-UC7 were pre-grown for 2 days in a YNBUP liquid medium containing 20 g/l glucose or xylose (Shi *et al.*, 1999). The cells were cultivated at 25 °C with shaking at 100 rpm. This is the standard xylose fermentation condition that we adapted to test our strains (Sreenath *et al.*, 1986; Cho and Jeffries, 1999). Cells were harvested and washed twice with sterile water. The cells were then used to inoculate 50 ml fresh YNBUP medium containing 84 g/l xylose or 94 g/l glucose in a 125 ml Erlenmeyer flask. Three replicates were run in each fermentation trial. The starting cell density was approximately 0.9 g/l. The cultures

were then grown at 23 °C with shaking at 100 rpm. Samples were drawn daily and analysed by HPLC as described above.

Cytochrome spectra study

Strains of *P. stipitis* were grown in medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l xylose, glucose or ethanol as the sole carbon source. The yeast cells were grown on the surface of a plate for 3 days as thin 'lines' and examined by a spectroscope as described by Sherman *et al.* (1974). Low temperature (– 196 °C) spectrophotometric recordings of the FPL-UC7 and FPL-Shi31 were performed with whole cells. The strains were grown on 10 g/l yeast extract, 20 g/l peptone and 20 g/l xylose at 30°C for 3 days. The absorption spectra were recorded as previously described (Hickey *et al.,* 1991).

Expression of *PsSTO1* in *S. cerevisiae*

To introduce *PsSTO1* into *S. cerevisiae,* the *PsSTO1* coding region was amplified using *Pfu* DNA polymerase from CBS 6054 genomic DNA. Primers 15 and 16 (not listed) were used, which contained a *Xho*I site at each end. The PCR conditions were described in Shi and Jeffries (1998). The amplified fragment was digested with *Xho*I, and cloned into the *Sal*I site of an expression vector, pYPR2831 (Horiuchi *et al.,* 1990) by using a Rapid DNA Ligation kit (Roche). Thus, the *PsSTO1* was driven by a strong constitutive glyceraldehyde 3-phosphate dehydrogenase (*ScTDH1*) promoter from *S. cerevisiae,* and terminated with a *TDH1* terminator. The resultant clone was named pNQ32. pNQ32 DNA (5 µg) was used to transform a *S. cerevisiae* strain, 679a (a gift from M. Culbertson). The empty vector without the *PsSTO1* expression cassette was also transformed into 679a and used as a control.

Analysis of the *S. cerevisiae* transformants containing *PsSTO1*

Primers 11 and 12 were used to confirm the transformants carrying the *PsSTO1* expression cassette. Transformants carrying the expression cassette were used for subsequent studies. For oxygen uptake experiments of 6790a transformants bearing either pNQ32 or pYPR2831, cells were grown in a 1 1 flask containing 500 ml YNBUP medium containing 75 g/l glucose at 30°C. The cultures were shaken at 140 rpm for 3 days. At the time of harvesting, the glucose present in the medium was completely consumed by the cells. The cells were washed twice with 20 mM potassium phosphate buffer (pH 7.0) containing 5 mM magnesium chloride, and suspended in 4.5 ml of the same buffer; 20 µl cells were used for each measurement. Duplicates were performed for each strain. For the 679a (pNQ32) strain, the cell amount (dry weight) used in each measurement was 7.3 mg while the cell amount (dry weight) used in each measurement for 679a (pYPR2831) was 6.8 mg. Oxygen uptake rates were measured as described above and the assay substrate was 5 mM ethanol.

Results

Cloning of the *PsSTO1* gene by genome walking

We introduced the genome walking method to clone *PsSTO1* from CBS 6054. Genome walking has been adapted to allow rapid cloning of a gene and its flanking regions from a known segment found in an unknown genome. To clone *PsSTO1,* we first need to identify a short segment of *PsSTO1* from the CBS 6054 genome. Alignment of the available fungal and yeast Sto protein sequences (Figure 2) revealed two highly conserved regions, IFLES(I/V)AGVPGMV and HRFVGYLEEEAV. The first region contains a putative ubiquinol binding site (Berthold, 1998; Andersson and Nordlund, 1999) while the second contains a conserved iron-binding motif (Moore *et al.,* 1995a, 1995b). These, along with a deduced codon usage table (Nakamura *et al.,* 2000) based on 19 cloned *P. stipitis* genes, were used to design degenerate primers. We amplified a 293 bp region, which is located in the centre of *PsSTO1,* from wild-type CBS 6054 genomic DNA. This segment displayed 70% similarity at the DNA level with the *STO* genes isolated from two closely related yeast species, *Pichia anomala* and *Candida albicans.* This 293 bp region was then used to design gene-specific primers for genome walking to clone the entire *PsSTO1* gene and its flanking regions.

The walking strategy is outlined in Figure 3A. Five linear fragment libraries (templates) were constructed as described in Methods. For the 5′ end walking, two overlapping fragments (1.7 kb and

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A.nidulans      ............................  MSTTGPIRVA AIPKHYLQFT VRTYTRSMAS AGLRYSNPPL VKKCYDQPTG KRFISSTPQS QIKDYFPPPD
A.niger         ........................... MN SLTATAPIRA AIPKSYMHIA TRNYSGVIAM SGLRCSGSLV ANRHQTA..G KRFISTTPKS QIKEFFPPPT
M.grisea        ................ MLVH QVNTKLCSAK QFTHLAKVVT PALSYQASSV YSANLPRLAA ................. SPR LFSTTSSAQL RDFFPVKETE
N.crassa        ........................ MNTPK VNILHAPGQA AQLSRALIST CHTRPLLLAG SRVATSLHPT QTNLSSPSPR NFSTTSVTRL KDFFPAKETA
A.Capsulatus    ............................ MY PTSGCARVLM ACPAPAMLRG PLLRPSTTAI RGLRGSPLLY HYAATSNSNM RYFSSTSRRW IKEFFAPPKE
C.albicans 1    MIGLSTYRNL PTLLTTTTVI STALRSKQLL RFTTT.TSTK SRSST.STAA TTVGNSNPK.  ..SPIDEDNL EKPGTIPTKH KP.. ..FNIQ TEVYNKAGIE
C.albicans 2    ................... M LTASLYKQLP VLTTATSTY SPIRLSSTLA TPPHSTTTTS PSSPAFHQPN HPQQFANPNT SV. ...FDVS TRIYTQEGID
P.anomala       ........................... MI KTYQYRSILN SRNVGIRFLK TLSPSPH ..................... SKD PNSKSIFDIG TKLIVNPPPQ
P.stipitis      ..........  ..MLSVQTTR AAKLQLGQLP LIAYTARSGR LHHQFYSTVA EKTANPT...  ................. PNT SDKTNIFDIR TKVYDETDIR
s.guttatum      ..........  MMSSRLVGTA LCRQLSHVPV PQYLPALRPT ADTASSLLHG CSAAAPAQRA GLWPPSWFSP PRHASTLSAP AQDGGKEKAA GTAGKVPPGE

A.nidulans      APKIVEVKTA WAHPVYSEEE MRAVTVGHRE AKNWSDWV..  ....................  ALGSVRLLRW GMDLVTGYKH PAPGQEDIKK FQ.......M
A.niger         APHVKEVETA WVHPVYTEEQ MKQVAIAHRD AKNWADWV..  ....................  ALGTVRMLRW GMDLVTGYRH PPPGREHEAR FK.......M
M.grisea        HIRQTPPTWP HHGLTEKEMV DVVPGHRKPR TLGDKFAW..  ....................  SLVRISRWGM DKVSGLSSEQ QQINKGSPTT SIVAA....K
N.crassa        YIRQTPPAWP HHGWTEEEMT SVVPEHRKPE TVGDWLAW..  ....................  KLVRICRWAT DIATGIRPEQ QVDKHH.PTT ATSAD....K
A.capsulatus    TDHIVESVTT WKHPVFTEKQ MKEIAIAHRE AKNWSDWV..  ....................  ALGTVRFLRW ATDLATGYRH AAPGKQGVEV PEQFQ. ...M
C.albicans 1    ANDDDKFLTK PTYRHEDFTE AGVYRVHVTH RPPRTIGD..  ....................  KISCYGTLFF RKCFDLVTGY AVPDPDKPDQ ..YKGTRWEM
C.albicans 2    NNNDTKFLTK APYPHPVFPQ DECENVTVTH RETKTLGD..  ....................  KISFRSIQFM RQCFDLVTGY AVPKTNNPDE ..FKGTRWEM
P.anomala       MA.DNQYVTH PLFPHPKYSD EDCEAVHFVH REPKTIGD..  ....................  KIADRGVKFC RASFDFVTGY KKPKDVNGML KSWEGTRYEM
P.stipitis      KHDDNQFITH PLFPHPTFSQ EDCLKVGYEH RPPRTFGD..  ....................  KMAFRGIELV RGSFDFVTGY KKPKTQADID SGFKGTRYEM
S.guttatum      DGGAEKEAVV SYWAVPPSKV SKEDGSEWRW TCFRPWETYQ ADLSIDLHKH HVPTTILDKL ALRTVKALRW PTDIFFQRRY ACRAMM.............

A.nidulans      ..TEKEWLRR FVFLESVAGV PGMVGGMLRH LRSLRRMKRD NGWIETLLEE AYNERMHLLT FLKMAEPGWF MRLHVLGAQG VFFNGFFLSY LISPRTCHRF
A.niger         ..TEQKWLTR FIFLESVAGV PGMVGGMLRH LRSLRRMKRD NGWIETLLEE AYNERMHLLT FLKLAEPGWF MRLMVLGAQG VFFNGFFLSY LMSPRICHRF
M.grisea        PLTEAQWLSR FIFLESIAAV PGMVAGMLRH LHSLRRLKRD NGWIETLLEE AYNERMHLLT FLKMCEPGWL MKILIIGAQG VYFNAMFVAY LISPKICHRF
N.crassa        PLTEAQWLVR FIFLESIAGV PGMVAGMLRH LHSLRRLKRD NGWIETLLEE SYNERMHLLT FMKMCEPGLL MKTLILGAQG VFFNAMFLSY LISPKITHRF
A.capsulatus    ..TERKWVIR FIFLETVAGV PGMVGGMLRH LRSLRRMKRD NGWIETLLEE AYNERMHLLS FLKLAQPGWP MRLMVLGAQG VFFNGFFISY LISPRTCHRF
C.albicans 1    ..TEEKWMTR CIFLESIAGV PGSVAGFVRH LHSLRMLTRD KAWIETLHDE AYNERMHLLT FIKIGKPSWF TRSIIYIGQG VFTNIFFLVY LMNPRYCHRF
C.albicans 2    ..TEGKWLTR CIFLESVAGV PGSVAGFLRH LHSLRMLRRD KAWIETLLDE AYNERMHLLT FIKIGKPSWF TRSIIYVGQG VFTNVFFLLY LLNPRYCHRF
P.anomala       ..TEEKWLTR CIFLESVAGV PGMVAAFIRH LHSLRLLKRD KAWIETLLDE AYNERMHLLT FIKIGNPSWF TRFIIYMGQG VFANLFFLVY LIKPRYCHRF
P.stipitis      ..TEGKWLTR CIFLESIAGV PGAVASFIRH LHSLRLLKRD KAWIETLLDE APNERMHLLT FIKIGKPSWF TRTIIYVGQG VFCNLFFLFY LANPKYCHRF
S.guttatum      ............ LETVAAV PGMVGGVLLH LKSLRRFEHS GGWIRALLEE AENERMHLMT FMEVAQPRWY ERALVLAVQG VFFNAYFLGY LLSPKFAHRV

A.nidulans      VGYLEEEAVL TYTRAIKDLE SGR.LPHW..  EKLEAPEIAV KYWKMPEGNR TMKDLLLYVR ADEAKHREVN HTLGNLKQAV DVNPFAVEWK DPSKPHPGKG
A.niger         VGYLEEEAVI TYTRAIKEIE AGS.LPAW..  EKTEAPEIAV QYWKMPEGQR SMKDLLLYVR ADEAKHREVN HTLGNLNQAI DPNPYAAKYK DPTKAHPNKG
M.grisea        VGYLEEEAVH TYTRSIEELE RGD.LPKWSD PKFQVPEIAV SYWGMPEGHR TMRDLLLYIR ADEANHRGVH HTLGNLNQVE DPNPFVSDYK GDKP..RPVA
N.crassa        VGYLEEEAVH TYTRCIREIE EGH.LPKWSD EKFEIPEMAV RYWRMPEGKR TMKDLIHYIR ADEAVHRGVN HTLSNLDQKE DPNPFVSDYK EGEGGRRPVN
A.capsulatus    VGYLEEEAVM TYTHAIKDLE SGK.LPNW..  ANQPAPDIAV AYWQMPEGKR TILDLLYYIR ADEAKHREVN HTLANLKQGV DPNPYAAKYD NPEAPHPTKS
C.albicans 1    VGYLEEEAVR TYTHLIDELD DPNKLPDF..  QKLPIPNIAV QYWPELTPES SPKDLILRIR ADEAKHREIN HTFANLEQWQ DRNPFALKIK DSDKPQPNYN
C.albicans 2    VGYLEEEAVR TYSHLLDELA VPGKLPAF..  ETMKIPEVAV QYWPELTPKS SPKDLILRIR ADEAKHREVN HTFANLEQKT DRNPFALKIE GLNKPQPNHG
p.anomala       VGYLEEEAVS TYTHLIKDID S.KRLPKF..  DDVNLPEISW LYWTDLNEKS TFRDLIQRIR ADESKHREVN HTLANLEQKK DRNPFALKVE DVPKEQQPDE
P.stipitis      VGYLEEEAVS TYTHFVHELQ S.GKLPKF..  ENIKIPTIAW QYWPELTENS SMLDLILRIR ADEAKHREVN HTLANLDQRK DRNPFGLAIP DL.KEPQPES
S.guttatum      VGYLEEEAIH SYTEFLKDID SGAIQDC...  ...PAPAIAL DYWRLPQGST LRDVVTVV.R ADEAHHRDVN HFASDVHYQD LELKTTPAPL GYH.......

A.nidulans      IKHLKTTGWE  REEVV.....   ....................
A.niger         IADLKPTGWE  REEVI.  ........................
M.grisea        AS.RPE.GFE REEVIGKEVI GKEVIEKDVI GKEVLGKQVS V
N.crassa        PA.LKPTGFE   RAEVIG  ........................
A.capsulatus    AEIVKPTGWE  RDEVI.  ........................
C.albicans 1    LDVTRPQGWE  RKDLYL....   ....................
C.albicans 2    INVMRPTGWE  KQDLQL..  .......................
P.anomala       YSLKTPHPEG  WNREQMRL..   ....................
P.stipitis      G.LKVTKPHG  WEKEELKL,.   ....................
S.guttatum      ......................................
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Figure 2. Sequence alignment of known fungal SHAM-sensitive terminal oxidase (Sto) proteins. The two isozymes from *Candida albicans* are listed as 1 and 2. The sequence of *Sauromatum guttatum* Sto is also listed because this was used as an antibody in the experiment described in Figure 6

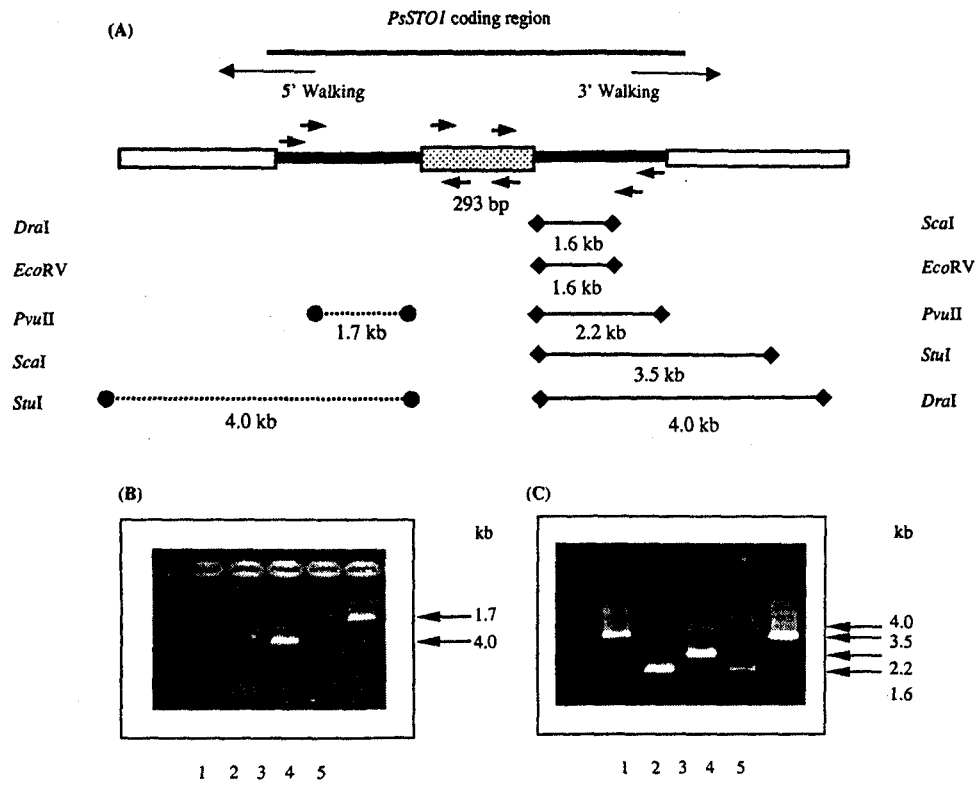

Figure 3. Cloning of *PsSTO1* from wild-type *Pichia stipitis* CBS 6054. (A) The genome walking strategy used to clone the *PsSTO1* gene. A 293 bp region (dotted box) in the middle of the *PsSTO1* gene was first amplified by using degenerate primers I and 2 (Table I). This region was then used as the starting point for walking towards both 5' and 3' ends. Solid bars represent the rest of the *PsSTO1* gene and white bars represent its flanking regions. (B) PCR products obtained from 5 linear fragment libraries during 5' walking (dotted lines). A 1.7 kb band was obtained from the *PvuII* library (lane 3). while a 4.0 kb fragment was amplified from the *StuI* library (lane 5). No products were obtained from the *DraI* library (lane I), the *EcoRV* library (lane 2) or the *SalI* library (lane 4). (C) PCR products obtained from five linear fragment libraries during 3′ walking (solid lines). A 3.5 kb band was obtained from the *StuI* library (lane I). A 1.6 kb band was obtained from the *ScaI* library (lane 2) and *EcoRV* library (lane 3). A 2.2 kb fragment was amplified from the *PvuII* library (lane 4). A 4.0 kb band was obtained from the *DraI* library. DNA sequences obtained from these segments allowed the assembly of the locus of *PsSTO1*

4 kb) were obtained from the *Pvu*II and the *Stu*I libraries (Figure 3B). For the 3′ end walking, five different lengths of overlapping fragments were obtained from the five linear libraries (Figure 3C). Sequencing of all the fragments allowed the merging of the coding region of 1071 bp, and about 0.9 kb each for the 5' and 3' flanking regions.

Characterization of the *PsSTO1* gene

The open reading frame of *PsSTO1* encodes for 357 amino acids. The deduced molecular weight for the premature protein is 41 326 kDa with a calculated pI of 9.1. These values resemble the numbers reported from other yeast and fungal Sto proteins (Sakajo *et al.,* 1993; Huh and Kang, 1999; Huh and Kang, 2001). The deduced cleavage site for the signal peptide was predicted at the 27th amino acid after the methionine start codon (Nielsen *et al.,* 1997). A search in the 5′ untranslated region for cis-acting elements (Wingender *et al.,* 2000) revealed two putative TATA boxes. One is located at −86to −79.the other is located

at −194 to −187. A putative 25 bp AT segment of mitochondrial control region (−158 to −133) is located between the two TATA boxes. Other putative binding sites included a common transcriptional activator responding to stress signals, Ap-1 (−689 to −683). a heat shock factor (hsp70p) and a nitrogen-regulatory element next to each other (−756 to −743). These observations indicated that the regulation of the *PsSTO1* gene could be mediated by various environmental signals. Because introns have been found in several fungal STO genes, we examined whether *PsSTO1* contains introns. RT−PCRwas performed on wild-type CBS 6054 cells grown on YNB-xylose. A single 1.1 kb band of RT−PCR product, which was similar in size to the genomic PCR product of *PsSTO1,* was observed (data not shown). Comparison of the cDNA and the genomic sequences showed that no intron was present in the *PsSTO1* gene. A phylogenetic study of all known Sto proteins from yeasts and fungi indicated that PsSto1p is most closely related to the Sto of Pichia anomala (Figure 2).

Construction of a *sto1*-D mutant in *P. stipitis*

To create a *sto1*-D mutant, we disrupted the cloned *PsSTO1* sequence by inserting the *PsURA3* cassette into the middle of the coding region (Figure 4A). The linearized disruption cassette was then used to transform our parental strain, FPL-UC7. Colony PCR indicated that the putative *sto1*-D mutants showed a single 2.4 kb band, which corresponded to the size of the coding region of *PsSTO1* plus the inserted *PsURA3* gene (Figure 4B). The parental strain, FPL-UC7, only showed a 1.1 kb band corresponding to the size of the coding region.

One of these *sto1*-D strains, designated FPL-Shi31, was first confirmed by measuring oxygen consumption rates in the presence of either antimycin A or SHAM. Upon adding 4 mM SHAM to the xylose-grown cells of FPL-Shi31, the respiration trace did not show any change (Figure 5A) and the cells exhibited insensitivity to SHAM. This suggested that the SHAM-sensitive respiration in this mutant was impaired due to the loss of the *PsSTO1* structural gene. In contrast, when antimycin A was added to the cells of the *sto1*-D mutant, the respiration rate dropped to zero (Figure 5B). This indicated that the mutant was unable to respire when the remaining CYT pathway was blocked. These observations were the opposite of the respiration traces from FPL-Shi21 (Figure 5C & 5D), a mutant

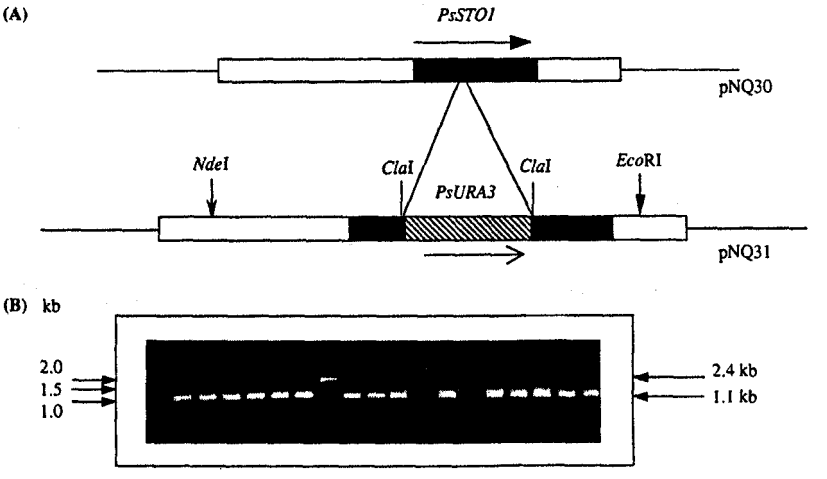

Figure 4. Disruption of the *PsSTO1* gene in *P. stipitis.* (A) A one-step gene replacement strategy was used to disrupt *PsSTO1* to create a knockout strain, FPL-Shi31 (*sto1*- D). The solid box represents the coding region of *PsSTO1* and the white box represents *PsSTO1* flanking regions in the plasmid pNQ30. The filled-in box represents the *PsURA3* cassette inserted into the *Cla*I site *PsSTO1* as pNQ31. Two restriction enzymes, *Eco*RI and *Nde*I. were used to linearize the disruption cassette to transform FPL-UC7 (*ura3-3*). (B) PCR screening of the putative *sto1*-D mutants. Arrows on the right indicate the sizes of PCR products

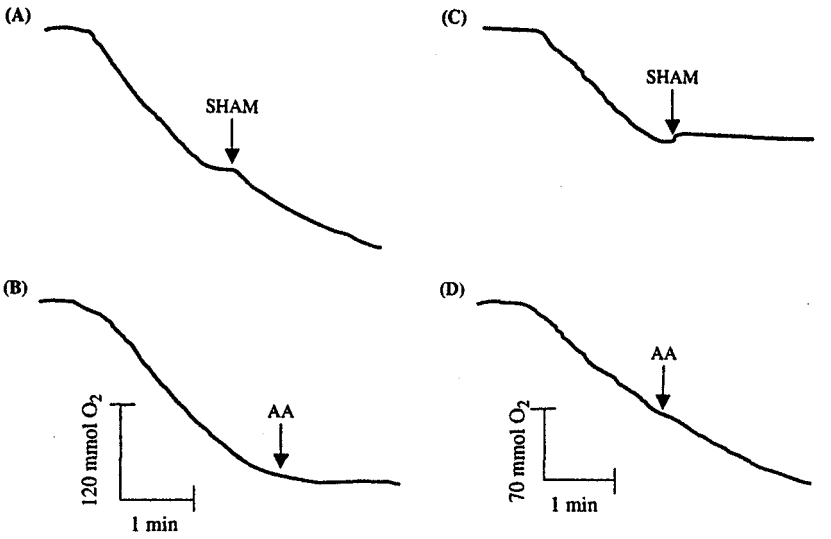

Figure 5. Confirmation of the *sto1*-D mutant (FPL-Shi31) by testing the oxygen uptake rates in the absence and presence of respiratory inhibitors. A *cyc1*-D mutant (Shi *et al.,* 1999) was used as the opposite strain for comparison. (A) Oxygen uptake trace for FPL-Shi31 cells in the absence and presence of 4 mM SHAM. (B) Oxygen uptake trace for FPL-Shi31 cells in the absence and presence of 10 μM antimycin A (C) Oxygen uptake trace for FPL-Shi21 cells in the absence and presence of 4 mM SHAM. (D) Oxygen uptake trace for FPL-Shi21 cells in the absence and presence of 10 μM antimycin A. Inhibitor solution was injected immediately into the chamber after adding the cells

containing a defective CYT pathway. Our data suggested that *PsSTO1* was disrupted in FPL-Shi31.

No other isoforms of Sto are present in the *sto1*-D mutant

To determine whether any other isoforms of Sto protein were present in FPL-Shi31 (*sto1*-D). mitochondrial proteins were isolated from four different strains of *P. stipitis.* A monoclonal antibody raised against a partially purified Sto protein from *Sauromatum guttatum* was used to cross-react with PsSto1p in the protein samples (Figure 6A). This monoclonal antibody has been shown to be able to cross-react with other Sto proteins or isoforms present in different tissues and growth stages from several fungal and plant species (Vanlerberghe and McIntosh, 1997). Thus, it is recognized as a reliable tool for detecting Sto proteins. In the wild-type CBS 6054 parental strain, FPL-UC7 and FPL-Shi21 (*cycl*-D), only a single 39 kDa band was observed (Figure 6B). This corresponded to the deduced size of the mature PsSto1p. However, this band was missing from the FPL-Shi31 (*sto1*-D) sample in which the *PsSTO1* gene was deleted. This result indicated that a single gene encodes the Sto protein in our parental strain, FPL-UC7. No other isoforms of Sto from the xylose-grown cells of FPL-Shi31 were able to cross-react with the monoclonal antibody raised against the Sto from *S. guttatum* (please see sequence in Figure 2). Given these findings and the results from the previous section, we confirmed that we had obtained a mutant in which the electron flow through PsSto1p was blocked.

Cytochrome spectra studies

We examined the cytochrome contents in the *P. stipitis sto1*-D mutant to determine whether any changes occurred after we deleted the structural gene of *PsSTO1.* In the initial studies, cells of the *sto1*-D mutant and the parental stain were cultivated in medium containing glucose, xylose or ethanol as a sole carbon source. Under spectromicroscopic examinations, cytochromes *b, c,* c_1 and $a{\cdot}a_3$ from the mutant cells grown on three media appeared to be at normal levels (data not shown).

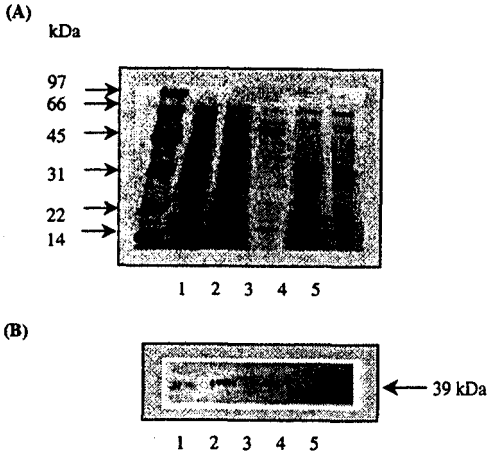

Figure 6. Western blot analysis on four *P. stipitis* strains. Mitochondrial proteins were extracted as described in Methods. Each lane contained approximately 15 µg protein. Sample from lane 3 (FPL-UC7) was obtained through an extra sucrose gradient purification and therefore the background for this sample is lower. Other samples were isolated by the mechanical method (Luttik *et al.,* 1998). Lane 1. wild-type CBS 6054; lanes 2 and 3, FPL-UC7 (*ura3-3*); lane 4, FPL-Shi31 (*sto1*- D); lane 5, FPL-Shi21 (*cyc1*- D). (A) The SDS–PAGE gradient gel for the mitochondrial proteins isolated from the four strains. One gel was stained with Coomassie blue while the other was used for electro-transfer to a nitrocellulose membrane. (B) The blot that contained the transferred proteins was first cross-reacted with a Sto monoclonal antibody (1:100 dilution) raised against *S. guttatum* (Elthon *et al.,* 1989). The blot was then hybridized with an anti-mouse secondary antibody conjugated with an alkaline phosphatase (1:5000 dilution). The colour was developed by a NBT/BCTP system (Promega). Arrow indicates the size of the mature PsSto1p protein

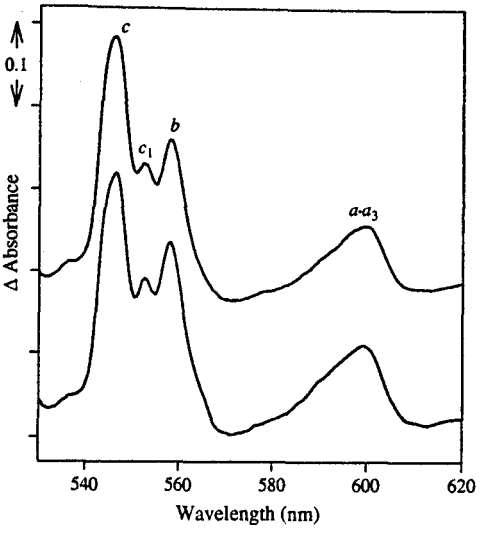

Figure 7. An example of the low temperature (–196°C) spectrophotometric recording of FPL-Shi31 (bottom curve) and the parental strain, FPL-UC7 (top curve). The cytochrome spectrum was determined in intact cells grown in medium containing 10 g/l yeast extract, 20 g/l peptone and 20 g/l xylose. The amounts and peak positions of cytochromes $a \cdot a_3$, b, c_1 and c from FPL-Shi31 were the same as the parent

Subsequent low temperature recordings were performed using xylose-grown cells from the mutant and the parent. One example is shown in Figure 7. If the STO system has cytochromes involved as functional components, their levels can fluctuate due to the loss of PsSto1p. We did not observe significant changes in any of the cytochrome levels in xylose-grown cells of FPL-Shi31. Thus, the deletion of PsSto1p does not change the cytochrome pool in the mutant.

Fermentation results

We then evaluated the performance of the *sto1*-D strain and its parent, FPL-UC7 in a YNBUP medium containing 84 g/l xylose or 94 g/l glucose as the sole carbon source. In the xylose fermentation trial, the mutant did not show significant difference in cell growth from the parent before 116 h. After 116 h, the mutant cells stopped growing while the parent continued to grow (Figure 8A). This suggests that PsSto1p plays a role in *P. stipitis* when grown under oxygen-limited conditions. Interestingly, the mutant produced 20% more ethanol (Figure 8A) and consumed xylose faster (Figure 8B) than the parental strain. The increase at ethanol production was observed starting at 46 h. The mutant accumulated xylitol slightly more rapidly than the parent, but the difference between the mutant and the parent was not so significant at the end of the fermentation (Figure 8B). Previous data demonstrated that SHAM-sensitive respiration is active in xylose-metabolizing cells and that it contributes to the total respiration in *P. stipitis* (Jeppsson *et al.,* 1995; Shi *et al.,* 1999). Therefore, deleting a component of the STO system, which is part of the respiration machinery in *P. stipitis,* should reduce respiration and lead to

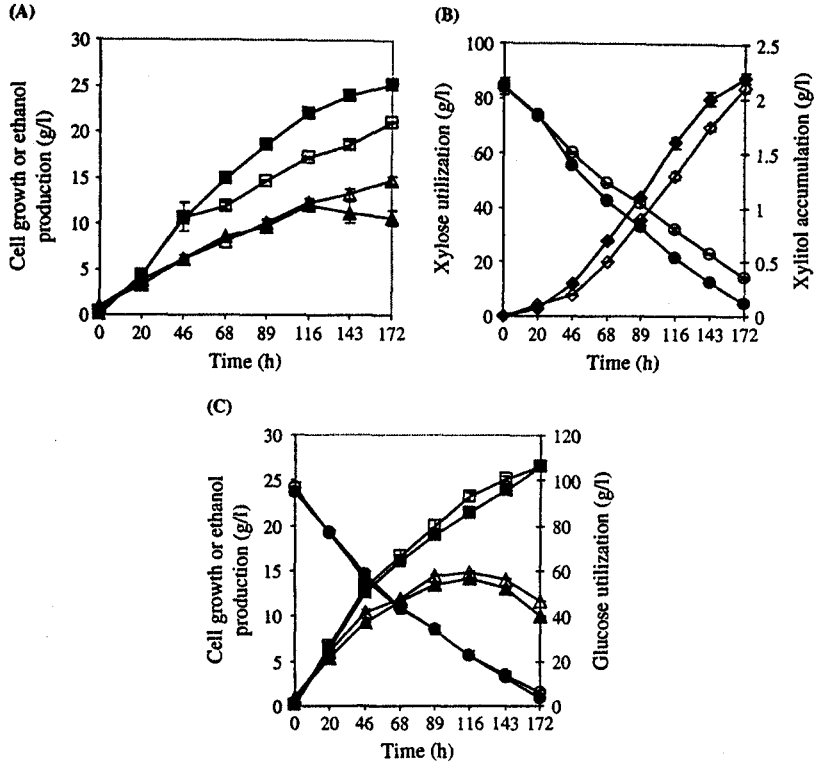

Figure 8. Xylose and glucose fermentation trials for FPL-Shi31 (*sto1*-D mutant) and FPL-UC7 (parent). Solid symbols represent data for the mutant, while open symbols represent data for the parent (A) and (B) contain data from the xylose fermentation trial. (A) Curves for cell yield and ethanol production. Solid triangle, cell yield for FPL-Shi31; solid square, ethanol production for FPL-Shi31; open triangle, cell yield for FPL-UC7; open square, ethanol production for FPL-UC7. (B) Curves for xylose utilization and xylitol accumulation. Solid circle, xylose utilization for FPL-Shi31; solid diamond, xylitol accumulation for FPL-Shi31; open circle, xylose utilization for FPL-UC7; open diamond, xylitol accumulation for FPL-UC7. (C) Curves for the glucose fermentation trial. Solid triangle, cell yield for FPL-Shi31; solid square, ethanol production for FPL-Shi31; solid circle, glucose utilization for FPL-Shi31; open triangle, cell yield for FPL-UC7; open square, ethanol production for FPL-UC7; open circle, glucose utilization for FPL-UC7

higher ethanol formation from xylose. In contrast, the mutant did not show any significant difference from the parental strain in cell growth, ethanol production or glucose utilization during the glucose fermentation trial (Figure 8C).

The presence of Complex I in *P. stipitis*

To further understand which component(s) at Site I of the ETC is linked to PsSto1p, we extended our studies to determine whether the NADH dehydrogenase complex (Complex I) and the independent NAD(P)H dehydrogenases are present in the mitochondria of *P. stipitis*. It has been documented that Crabtree-negative yeasts possess independent NADH dehydrogenases in addition to Complex I in their mitochondria (Ohnishi, 1972, 1973). A respiratory inhibitor, rotenone, was added to the glucose- or xylose-grown cells of wild-type CBS 6054 to distinguish the presence of Complex I or the independent NAD(P)H dehydrogenases. Complex I is sensitive to rotenone, while the independent NAD(P)H dehydrogenases are insensitive to it. In the absence of rotenone, oxygen consumption rates by *P. stipitis* CBS 6054 cells grown aerobically on xylose or glucose were detected (Table 3). When 0.1 mM rotenone was added to

Table 3. Oxygen consumption rate of wild-type *P. stipitis* CBS 6054 in the presence or absence of 0.1 mM rotenone

Glucose–grown cells (μmol/mg/h)		Xylose–grown cells (μmol/mg/h)	
– Rotenone	+ Rotenone	– Rotenone	+ Rotenone
2.9 ± 0.1*	0	4.5 ± 0.2	4.6 ± 0.2

*Standard deviation was calculated from at least two independent measurements

the glucose-grown cells of CBS 6054, the cells stopped respiring immediately. In contrast, when the same concentration of rotenone was injected to the xylose-grown cells of CBS 6054, respiration was sustained and the rate was similar to that of the untreated cells. These observations provided initial evidence that Complex I is present in the mitochondria of *P. stipitis* but it is bypassed in the xylose-grown cells.

We further tested the rotenone sensitivity in the xylose-grown cells of the *sto1*-D mutant (FPL-Shi31) and its parent, FPL-UC7 by adding rotenone to the cells, as described previously. In contrast to the parent cells, the *sto1*-D mutant, which had lost PsSto1p, were inhibited by rotenone.

Expressing *PsSTO1* in *S. cerevisiae*

The purpose of expressing *PsSTO1* into the Crabtree-positive *S. cerevisiae* was twofold. First, we needed to ensure that the introduced PsSto1p, like other reported Sto proteins, could confer cyanide-resistant oxygen uptakes in a heterologous host. Second, we hoped to see if we could modify the respiratory flux in *S. cerevisiae* by adding an additional electron acceptor to its ETC. Moreover, we could use *S. cerevisiae,* which has a relatively simpler respiratory network (Complex I, the independent NADPH dehydrogenases and Sto are absent), to determine whether PsSto1p itself is coupled to ATP synthesis.

We constructed the *PsSTO1* expression cassette and transformed a *S. cerevisiae* host, 679a. Colony PCR was used to confirm that the transformants bearing either the pNQ32 plasmid or the empty vector, pYPR2831, in 679a (data not shown). For oxygen uptake measurements, we grew both 679a (pNQ32) and 679a (pYPR2831) strains in YNBUP medium containing 75 g/l glucose, as described elsewhere. At the time of harvest, glucose had been completely consumed by both strains. We observed no significant difference in ethanol formation between cells with *PsSTO1* overexpressed and the control cells. Overexpression of *PsSTO1* resulted in higher accumulation of glycerol (4.3 g/l vs. 2.6 g/l for the control). This difference suggested that the respiratory flux was modified in the strain with *PsSTO1* overexpressed. We then grew both strains in 25 ml YNBUP medium containing 20 g/l ethanol as the sole carbon source in a 125 ml Erlenmeyer flask. The cells were shaken at 140 rpm at 30°C. The control cells grew to approximately 10 OD_{600} after 3 days, while the cells with *PsSTO1* overexpressed did not show significant growth.

We then measured oxygen consumption rates from the 679a (pNQ32) cells and the control using ethanol as the substrate. Cells with *PsSTO1* overexpressed showed 63% higher oxygen consumption rate than the control st`rain (Figure 9). We measured the total respiration rate in the presence of KCN as well. Cyanide is a respiratory inhibitor that blocks electron transfer from cytochrome *c* to cytochrome *c* oxidase (Figure 1). Upon adding 1 mM KCN to the cells, the 679a (pNQ32) strain containing *PsSTO1* showed a fivefold higher cyanide-resistant oxygen consumption rate than the control strain. We observed a similar result when 10 μM antimycin A was used as the inhibitor (data not shown). These results suggested that the introduced PsSto1p was able to confer cyanide-resistant respiration in the cells with *PsSTO1* overexpressed. Moreover, these cells showed increased total oxygen uptake. Our observations are in agreement with previous reports on other expression studies of the SHAM-sensitive alternative oxidase genes from *Candida albicans* in *S. cerevisiae* (Huh and Kang, 1999, 2001), and an alternative oxidase gene of *S. guttatum* in *Schizosaccharomyces pombe* (Affourtit *et al.,* 1999). Our observations indicated that *PsSTO1,* when expressed in *S. cerevisiae,* was able to increase oxygen consumption in the host cells but did not lead to growth on ethanol. This suggests that PsSto1p itself is not coupled to ATP synthesis.

Discussion

SHAM-sensitive alternative respiration is a common phenomenon reported in the mitochondria of

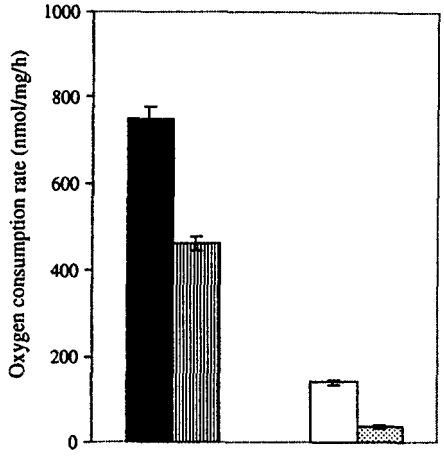

Figure 9. Oxygen uptake measurement in *S. cerevisiae* 679a strain containing *PsSTO1*. *PsSTO1* was driven by a *ScTDH1* promoter and terminated by a *ScTDH1* terminator. Preparation of cells and measurement of oxygen consumption were as described in Methods. Bars on the left are total respiration rates detected from the transformed cells in the absence of KCN. solid bar, 679a (pNQ32); lined bar, 679a (pYPR2831)(control). Bars on the right are total respiration races detected from the transformed cells in the presence of I mM KCN: open bar, 679a (pNQ32): dotted bar, 679a (pYPR2831)(control)

many yeasts, fungi and higher plants. Although intensive studies in plant mitochondria have shed light on this interesting system, it is rather difficult to further address the pathway components and functional roles of STO using plants. Yeasts are better alternatives because one can create mutants in various respiratory systems and easily measure cell yield, oxygen uptake and metabolite formation. To improve our understanding of the physiological functions of the STO system, we cloned and disrupted the *PsSTO1* gene in a Crabtree-negative yeast, *P. stipitis.*

It has been known that both CYT and STO respiration systems are present in wild-type *P. stipitis* during xylose conversion to ethanol (Jeppsson *et al.,* 1995; Shi *et al.,* 1999; Shi, 2000). Moreover, the transcriptional level of the cytochrome *c* gene in wild-type CBS 6054 is downregulated during xylose fermentation (Cho and Jeffries, 1999). This indicates that the CYT pathway is suppressed when oxygen is limited due to high cell density. In our xylose fermentation trial, the control cells continued to grow, while the growth of the *sto1*-D mutant stopped after 116 h. This indicated that the mutant cells were unable to cope with the low-oxygen environment after losing PsSto1p, especially at the end of our fermentation when cell densities were high. It has been reported previously that a plant Sto homologue shows higher affinity for oxygen than does Cox (Lambert, 1982). PsSto1p may play a role in scavenging for residual oxygen when the CYT system is suppressed under oxygen-limited conditions. A functioning STO pathway allows the cells to at least maintain electron transfer (not necessarily to generate ATP). Such a system could be very useful for Crabtree-negative yeasts and fungi to sustain respiratory flux when the standard CYT system is perturbed by environmental stresses.

In our studies, we could not completely rule out the possibility that PsSto1p is coupled to ATP synthesis in the background of *P. stipitis.* We then expressed *PsSTO1* in *S. cerevisiae,* which has a relatively simpler ETC. Thus, we obtained a new strain containing two electron acceptors, Cox and PsSto1p. We observed no respiratory growth on ethanol from this strain but did detect an increased rate of oxygen consumption. This suggests that the introduced PsSto1p itself is not proton-motive. Moreover, the introduced PsSto1p can accept electrons from the independent NADH dehydrogenases (Nde and Ndi) or succinate dehydrogenase complex. Because these dehydrogenases are non-proton-translocating, no respiratory growth would be observed on ethanol. This demonstrates that the connection of PsSto1p to these dehydrogenases can form a system that is not linked to ATP synthesis.

Following this observation, we studied the origin of electrons (Site I) donated to PsSto1p during xylose conversion in the cells of *P. stipitis.* It has been documented that sensitivity to rotenone can be used to distinguish the presence of Complex I or the independent NAD(P)H dehydrogenases at Site I (Ohnishi *et al.,* 1972; Ohnishi *et al.,* 1973). We first performed the study in xylose- and glucose-grown cells of wild-type CBS 6054. We observed that rotenone blocked oxygen uptake in glucose- but not in xylose-metabolizing cells. This indicated that wild-type cells employ Complex I during glucose metabolism, whereas Complex I was bypassed in xylose-metabolizing cells under our tested conditions. These observations imply that the rotenone-insensitive NAD(P)H dehydrogenases are present in the cells during xylose metabolism. Therefore, only two proton-translocating sites (III

and IV) that are associated with ATP synthesis are functioning in the ETC when the cells metabolize xylose. This led us to postulate that the rotenone-insensitive NAD(P)H dehydrogenases could link to PsSto1p in the cells during xylose conversion. Such connection(s) would form a system consisting completely of alternative components that are not coupled to ATP synthesis. Joseph-Home et al. (2001) suggested that this system would be avoided in yeasts and fungi because it only maintains electron flux but does not lead to generation of ATP.

So what can such a system do? It may serve a regulatory function in the redox network present in the mitochondria of Crabtree-negative species. According to Lambert's (1982) overflow hypothesis on the STO system present in plant mitochondria, the standard CYT pathway can be saturated, which results in increased ratios of ATP/ADP and $NADH/NAD^+$ in the cell. Under such physiological conditions, the TCA cycle slows down, which limits the production of carbon skeletons. The operation of the STO system may help increase carbon flow in the cell. On the other hand, the suppression of the STO system can allow the TCA cycle to 'spin off carbon skeletons. Thus, such a non ATP-generating system might be very useful to Crabtree-negative yeasts when they need to deal with carbon-excess conditions.

We further detected that the xylose-metabolizing cells of the *sto1*-D mutant became rotenone-sensitive after losing PsSto1p. This suggests that Complex I is somehow employed by the mutant. This could be due to the loss of the electron receptor, PsSto1p, for the alternative system. It has been well documented in bacteria (Gennis and Stewart, 1996) and higher plants (Douce and Neuburger, 1989) that the components from the alternative and standard respiration systems can be interchangeable. The cells always select the most efficient system to produce just enough energy to support growth and other cellular activities. Complex I in higher eukaryotes consists of more than 40 subunits. It pumps protons from the oxidation of matrix NADH across the mitochondrial inner membrane (Rasmusson *et al.,* 1998). The 'activation' of Complex I in the mutant cells of *sto1*-D during xylose metabolism implies that PsSto1p is part of the machinery that supports NADH oxidation. We did not observe increased cell yield from the xylose-grown cells of the *sto1*-D mutant (with an 'activated' Complex I). This could be related to how well Complex I functions under oxygen-limited conditions in *P. stipitis,* which has not been extensively studied, and the concentration of NADH in the cell at this physiological state.

We observed fundamental differences between the respiratory machinery supporting xylose and glucose metabolism. In contrast to the xylose-grown cells, the glucose-grown cells of *P. stipitis* were sensitive to rotenone, which indicates that Complex I is employed in those cells when metabolizing glucose. This suggests that at least three proton-translocating sites linked to ATP synthesis are present in the glucose-grown cells. In wild-type *P. stipitis* CBS 6054, Ligthelm (1987) showed that the maximum growth rate (μ_{max}) on glucose was twofold higher than that on xylose under fermentative conditions. Interestingly, Skoog (1992) reported that the ATP:ADP ratio was higher from glucose-grown CBS 6054 cells than from xylose-grown cells when both were cultivated aerobically. These data provided the basis to support our observations. Further investigation is needed to elucidate the physiological roles of the STO system in the glucose-metabolizing cells of ***P. stipitis.*** Our future studies will provide new insights into the complex redox networks present in Crabtree-negative yeasts and fungi.

Acknowledgements

The authors sincerely thank Daniel Cullen for his helpful suggestions on genome walking, Philip Kirsten and Kent Hammel for use of equipment Thomas Cardillo and David Pearce for their assistance in the cytochrome spectrophotometric recordings, Tom Elthon for the Sto monoclonal antibody of *S. guttatum*, Michael Culbertson for the *S. cerevisiae* host 679a and Debora Berthold, Won-Ki Huh, George Hill and Minu Chaudhuri for sharing valuable information in their alternative oxidase research projects. We are also grateful to Hans van Dijken for his stimulating discussions. This work was supported by National Renewable Energy Laboratory Subcontract XAU-4-11193-02 and USDA NRICGP Grant 96-35500-3172 to T.W.J.; by National Institutes of Health Grant ROI GM12702 to F.S.; by Ruth Dicky Memorial Research Awards to N.Q.S.; and by a Research Travel Grant from the University of Vigo to J.C.

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