Physiological role of the glutathione-dependent formaldehyde dehydrogenase in the methylotrophic yeast *Candida boidinii*

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The methylotrophic yeast *Candida boidinii* exhibits formaldehyde dehydrogenase activity (FLD, EC 1.2.1.1) during growth on methanol as a sole carbon source. The structural gene, *FLD1*, was cloned from a genomic library of *C. boidinii*. The 1263 bp *FLD1* gene contained a 123 bp intron and its exon encoded a gene product of 380 amino acids, whose predicted amino acid sequence showed high similarity to the sequences of FLDs from other organisms. The *FLD1* gene was disrupted in the *C. boidinii* genome by one-step gene disruption. The *fld1* Δ strain could not grow on methanol as a carbon source under methanol-limited chemostat culture conditions, even with low dilution rates ($D < 0.05 \text{ h}^{-1}$), whereas a strain with a disruption in the gene for formate dehydrogenase (FDH; another NADH-generating dehydrogenase involved in the formaldehyde oxidation pathway) could survive. These results indicated that FLD, but not FDH, is essential for growth of *C. boidinii* on methanol.

Keywords: formaldehyde oxidation pathway, gene cloning, gene disruption, energy generation, formaldehyde detoxification

INTRODUCTION

Methylotrophic yeasts are able to use methanol as a sole carbon and energy source. In yeast methanol metabolism, methanol is first oxidized to formaldehyde by alcohol oxidase (AOD; EC 1.1.3.13), which is localized in peroxisomes (Sakai & Tani, 1992b). Then, formaldehyde enters both the assimilation and dissimilation pathways (Anthony, 1982). In the assimilation pathway, formaldehyde is fixed to xylulose 5-phosphate by peroxisomal dihydroxyacetone synthase (DHAS; EC 2.2.1.3). In the dissimilation pathway, formaldehyde generated within peroxisomes non-enzymically reacts with reduced glutathione to form S-hydroxymethylglutathione. Then, S-hydroxymethylglutathione formed in peroxisomes is exported to the cytosol (Horiguchi *et al.*, 2001), where it acts as a substrate for glutathione-dependent formaldehyde dehydrogenase (FLD; EC 1.2.1.1). S-Formylglutathione produced by FLD is then

hydrolysed to glutathione and formate by S-formylglutathione hydrolase (EC 3.1.2.12). Finally, formate is oxidized to carbon dioxide by formate dehydrogenase (FDH; EC 1.2.1.2). During this formaldehyde oxidation pathway, NADH, as an energy source, is generated through two reactions catalysed by NAD⁺-dependent dehydrogenases, FLD and FDH (Veenhuis *et al.*, 1983).

Formaldehyde is a highly reactive compound that has a toxic effect on all organisms through its nonspecific reactivity with proteins and nucleic acids (Feldman, 1973; Grafstrom *et al.*, 1983). Since formaldehyde is located at the branching point of the assimilation and dissimilation pathways, proper maintenance of the formaldehyde level is an important factor for efficient C₁ metabolism. The dissimilatory formaldehyde oxidation pathway is thought to play a significant role in the detoxification of formaldehyde.

There have been some discrepancies in reports regarding the ability of FDH-defective mutants of methylotrophic yeasts to grow on methanol. Bystrykh *et al.* (1988) reported that an FDH-deficient mutant of *Hansenula polymorpha* could not grow on methanol. On the other hand, Sibirny *et al.* (1990) reported that both FLD- and FDH-defective mutants of *H. polymorpha* could grow

Abbreviations: AOD, alcohol oxidase; DHAS, dihydroxyacetone synthase; FDH, formate dehydrogenase; FLD, glutathione-dependent formaldehyde dehydrogenase.

The GenBank accession number for the sequence reported in this paper is AB085186.

on methanol, and suggested that neither FLD nor FDH was essential for the energy supply for growth on methanol. Recently, a *Pichia pastoris* FLD-defective mutant was reported to be unable to grow on methanol (Shen *et al.*, 1998). We think that these discrepancies are due to the following. (1) The previous studies used mutants derived via chemical mutagenesis, and these mutant strains could exhibit leaky phenotypes. (2) Both batch and chemostat culture conditions were confusingly used for the growth analyses, leading to misevaluation of the growth ability of the mutants.

We have been using *Candida boidinii* as a model organism to study the physiological role of methanol-metabolizing enzymes (Horiguchi *et al.*, 2001; Sakai *et al.*, 1996, 1997, 1998). One of the merits of using *C. boidinii* is the ease with which gene-disrupted strains can be derived (Sakai & Tani, 1992a). Previously, we showed that an enzyme involved in the formaldehyde oxidation pathway, FDH, plays not an essential but a significant role during growth on methanol (Sakai *et al.*, 1997), using an *FDH1*-disrupted strain of *C. boidinii* in combination with the methanol-limited chemostat technique. In a methanol-limited chemostat culture with a low dilution rate ($0.03\text{--}0.05\text{ h}^{-1}$), the *fdh1Δ* strain could grow on methanol as a sole carbon source, although the growth yield was diminished compared with that of the wild-type strain (Sakai *et al.*, 1997). These previous results raised the possibility that the formaldehyde oxidation pathway is not essential for growth on methanol of *C. boidinii*, although it could contribute significantly to the energy yield during growth on methanol. In this study, we focus on another NADH-generating dehydrogenase involved in the formaldehyde oxidation pathway, FLD. The physiological role of FLD was investigated through cloning, gene disruption, and expression analyses of the FLD-encoding gene in *C. boidinii*, in combination with chemostat-culture techniques. The results obtained showed that the glutathione-dependent formaldehyde oxidation pathway is indeed necessary for growth on methanol of *C. boidinii*.

METHODS

Yeast and bacterial strains, media, and cultivation. *C. boidinii* S2 (Tani *et al.*, 1985) was used as the wild-type strain, and as the source of FLD and chromosomal DNA. *C. boidinii* TK62 (*ura3*) (Sakai *et al.*, 1991) was used for transformation. *Escherichia coli* JM109 was used for plasmid propagation and construction of a *C. boidinii* S2 genomic library.

MI medium was used as the basal medium (Sakai *et al.*, 1991), with the carbon and nitrogen sources as follows: 1.5% (v/v) methanol, 3% (v/v) glycerol, 2% (w/v) glucose, 0.76% (w/v) NH_4Cl , 0.5% (w/v) methylamine hydrochloride, 0.5% (w/v) choline chloride and 1 mM formaldehyde. The initial pH of the medium was adjusted to 6.0. Cultivation was performed on a shaking incubator at 28 °C and growth was monitored by measuring optical density at 610 nm. The formaldehyde was monitored by the method of Nash (1953).

E. coli was grown at 37 °C in 2× YT medium supplemented, when necessary, with ampicillin ($50\text{ }\mu\text{g ml}^{-1}$).

Protein methods and enzyme assays. Cells were suspended in 0.1 M potassium phosphate buffer, pH 7.0, and then transferred to a 2 ml Eppendorf tube containing an equal volume of 0.5 mm zirconium beads. The tube was shaken vigorously for 30 s with a mini-beadbeater model 693 (Biospec Products), and then chilled on ice for 30 s. This procedure was repeated six times, and the cell debris was removed by centrifugation at 16000 g for 5 min at 4 °C. The resultant supernatant was immediately used for enzyme activity assays. FLD activity was measured by determining the rate of NADH formation at 340 nm at 30 °C as described previously (Schutte *et al.*, 1976). One unit of enzyme activity was defined as the amount of enzyme which produced $1\text{ }\mu\text{mol NADH min}^{-1}$. Protein was determined by the method of Bradford (1976) with a protein assay kit (Bio-Rad) and bovine serum albumin as the standard. SDS-PAGE was performed with a polyacrylamide slab gel and a Tris/glycine buffer system as described by Laemmli (1970).

Purification of FLD. Methanol-grown cells of *C. boidinii* were harvested and washed with 50 mM Tris/HCl buffer (pH 8.5). The cells were disrupted with a 200M Insorator (KUBOTA), and then the cell debris was removed by centrifugation at 20000 g. The resultant supernatant was applied to a DEAE-Sephacel column ($5.0\times 15\text{ cm}$) equilibrated with 50 mM Tris/HCl buffer (pH 8.5). After washing of the column with the same buffer, elution was carried out with a gradient of 0 to 0.5 M KCl in 50 mM Tris/HCl buffer (pH 8.5). The active fractions were pooled, concentrated, and subsequently saturated with 3.0 M ammonium sulfate. The enzyme was then applied to a Butyl-Toyopearl column ($2.2\times 20\text{ cm}$) equilibrated with 3.0 M ammonium sulfate in 50 mM Tris/HCl buffer (pH 8.5). After washing of the column with the same buffer, elution was performed with a gradient of 3.0 to 0 M ammonium sulfate. The active fractions were pooled, concentrated, and subsequently applied to a Superdex 200 column ($1.6\times 60\text{ cm}$) equilibrated with 50 mM Tris/HCl buffer (pH 8.5).

Determination of partial amino acid sequences. The purified enzyme (1.2 mg) was digested with *Achromobacter* lysyl endopeptidase at an enzyme:substrate ratio of 1:200 for 12 h at 37 °C in 0.1 M ammonium bicarbonate, and the resulting peptide mixture was separated by reverse-phase HPLC on a column of Cosmosil 5C18-AR300 ($4.6\text{ mm}\times 25\text{ cm}$; Nacalai Tesque). Gradient elution was performed at 0.5 ml min^{-1} with 0.06% trifluoroacetic acid in water as solvent A and 0.052% trifluoroacetic acid in 80% acetonitrile as solvent B. The amino acid sequences of the amino-terminal regions of the purified enzyme and peptides were determined with a protein analyser (Applied Biosystems model 4701A) on an on-line HPLC apparatus (model 120A).

DNA methods. Yeast DNA was purified by the method of Cryer *et al.* (1975) or Davis *et al.* (1980). Southern analysis was performed essentially as described previously (Yurimoto *et al.*, 2000a). Transformation of *C. boidinii* TK62 (*ura3*) was performed by the modified lithium acetate method, as described previously (Sakai *et al.*, 1993). pBluescript II SK+ and pBluescript KS− were from Stratagene, pT7Blue was from Novagen and pUC118 was from New England BioLabs. DNA was sequenced with a 7-deaza sequencing kit (Thermo sequence fluorescent labelled primer cycle sequencing kit) from Amersham Pharmacia Biotech and a DNA sequencer model DSQ-2000L from Shimadzu.

Cloning of the *C. boidinii* FLD1 gene. PCR was carried out to obtain a DNA probe for screening the gene library via colony hybridization. The oligonucleotide primers were designed on

Fig. 1. (a) The amino acid sequences determined from purified FLD (LF-1 to LF-6). Arrows indicate the oligonucleotides used for gene cloning. (b) Intron sequence (bold underline) of the *FLD1* gene. The nucleotide and amino acid numbering starts from the initiator ATG.

the basis of internal partial amino acid sequences determined from purified FLD (Fig. 1a, LF-1 to LF-6). According to the amino acid sequences YTPECREC from LF-3 and AFHDMHAG from LF-4, a forward primer (primer 1; a 192-fold redundant 23-mer [5'-TAYACHCCWGAAWSIMGYG-AAGC-3']) and a reverse primer (primer 2; a 144-fold redundant 23-mer [5'-CCDGCRGTGCATRTCTRTGRAADGC-3']) were designed and synthesized. The PCR reaction

mixture consisted of 0.35 µg *C. boidinii* S2 genomic DNA as the template, 0.5 µg of each mixed primer, 0.2 mM dNTPs, 50 mM KCl, 10 mM Tris/HCl buffer (pH 8.3), 1.5 mM MgCl₂, 0.001% (w/v) gelatin and 2.5 U *Ex Taq* DNA polymerase (Takara Shuzo) in a total volume of 100 µl. PCR was performed with a Perkin-Elmer model 480 DNA thermal cycler under the following temperature profile conditions: denaturation, 95 °C, 1 min; annealing, 56 °C, 0.5 min; extension, 72 °C, 1.5 min; for 30 cycles. The amplified 0.9 kb PCR fragment was ligated to the pT7Blue vector and then introduced into *E. coli* JM109 cells. The 0.9 kb *Nde*I–*Bam*HI fragment of the recombinant plasmid was gel-purified and used as a probe for hybridization experiments. The partially *Sau*3AI-digested genomic DNAs were ligated into *Bam*HI-digested and dephosphorylated pUC118. To construct a *Sau*3AI gene library, *E. coli* JM109 cells were transformed with the resulting ligation mixture. Colonies formed on the master plates were transferred to Biodyne nylon membranes (Pall Bio Support). After lysis of *E. coli* and binding of the liberated DNA to the nylon membranes, the blots were used for colony hybridization by the Church and Gilbert hybridization method (Church & Gilbert, 1984). Hybridization was performed overnight at 65 °C, and then the membranes were washed three times in 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at the same temperature. Positive clones were picked up from the master plates and used for further studies (pFL2).

Northern analysis. Total RNAs were extracted from *C. boidinii* cells grown on various carbon and nitrogen sources using ISOGEN (Nippon Gene), and then electrophoresed on a 0.8% agarose gel made with 20 mM MOPS buffer containing 1 mM EDTA and 2.2 M formaldehyde. Hybridization was performed under high-stringency conditions with a ³²P-labelled probe of a 0.9 kb PCR fragment of the *C. boidinii*

Fig. 2. Physical map of the cloned *FLD1* gene and its one-step disruption in the *C. boidinii* genome. (a) Restriction map of the cloned fragment and the gene-disruption strategy. The shaded boxes at the ends of *URA3* show the repeated sequences for homologous recombination to remove the *URA3* gene after gene disruption. (b) Genomic Southern analysis of *Hind*III-digested total DNAs (3 µg of each): lane 1, the host strain TK62; lane 2, the *fld1*Δ strain; lane 3, the *fld1*Δura3 strain, probed with the ³²P-labelled 1.0 kb PCR fragment including the 5'-flanking region of *FLD1*.

FLD1 coding region or a 0.9 kb *ClaI*–*HindIII* fragment harbouring *C. boidinii* *ACT1* DNA (encoding actin) (Sakai *et al.*, 1996). The size of the hybridizing band was estimated from the mobilities of the 0.25–9.5 kb RNA ladder (Life Technologies).

Construction of an *FLD1* gene disruption cassette and one-step gene disruption. To remove an approximately 0.5 kb fragment including most of the coding sequence of *FLD1*, the 1.0 kb *SacI*–*Bam*HI fragment derived from pFL2 was PCR-amplified using a pair of primers, forward primer [5'-CTGgagctcGCTATTATCAGTGTATTTTAAT-3'] and reverse primer [5'-CGggatccACGACATTCTGGAGTGTA-3'], and the other part of the 2.0 kb *Bam*HI–*Pst*I fragment derived from pFL2 was PCR-amplified using another pair of primers, forward primer [5'-CGggatccATTGACATGGACCGATGG-3'] and reverse primer [5'-AActgcagGTGCTGTGTACGGA-TGTT-3'] (restriction sites are shown in lower case). These two fragments were ligated to *SacI*–*Pst*I-digested pBluescript II SK+. The plasmid obtained was digested with *Bam*HI, blunt-ended, and then ligated with the blunt-ended *SalI*–*XhoI* fragment derived from pSPR, which contained the *C. boidinii* *URA3* gene with repeated flanking sequences (Sakai & Tani, 1992a). The resulting disruption vector was digested with *SacI* and *Pst*I, and then used for transformation of *C. boidinii* strain TK62. The disruption of the *FLD1* gene (yielding the *fld1Δ* strain) and loss of the *URA3* gene (yielding the *fld1Δura3* strain) were confirmed by genomic Southern analysis of *HindIII*-digested DNA from the transformants, using the 1.0 kb *SacI*–*Bam*HI fragment from pFL2 as a probe (Fig. 2).

RESULTS

Cloning of *C. boidinii* *FLD1* and its primary structure

FLD was purified by column chromatography to apparent homogeneity, as judged from the single band on SDS-PAGE (see Methods). The partial amino acid sequences of six internal peptide fragments were determined, but the N-terminal seemed to be blocked. Synthetic oligonucleotides, primer 1 and primer 2, were designed based on the amino acid sequences of internal peptide sequences LF3 and LF4, respectively (Fig. 1a). The PCR reaction, using these synthetic primers and the *C. boidinii* genomic DNA as a template, amplified a 0.9 kb fragment. One of the deduced amino acid sequences of this amplified fragment showed high similarity to the amino acid sequences of FLDs from other sources (Shen *et al.*, 1998). The *Sau3AI*–*C. boidinii* gene library was screened by colony hybridization-selection using this 0.9 kb ³²P-labelled probe. From among a total of approximately 22000 independent colonies obtained, eight positive clones were isolated. Southern hybridization analysis of the plasmids recovered from these positive clones revealed that the 0.9 kb probe hybridized with a 3.5 kb *Bam*HI–*Pst*I fragment of the plasmid (Fig. 2). Sequence analysis revealed an ORF in the 3.5 kb fragment, which contained identical amino acid sequences to ones determined for the purified FLD (Fig. 1a). Based on these and the following observations, we concluded that this ORF encodes the gene for FLD in *C. boidinii* (*FLD1*).

The *FLD1* ORF consisted of two exons (encoding 6 and 368 amino acids, respectively), separated by one 123 bp

Fig. 3. Regulation of *FLD1* expression in *C. boidinii*. (a) FLD activities in extracts of cells grown on the indicated carbon and nitrogen sources. The carbon sources were as follows: column 1, methanol; columns 2, 3 and 4, glucose; columns 5, 6 and 7, glycerol; column 8, glucose+formaldehyde. The nitrogen sources were as follows: columns 1, 2, 5 and 8, NH₄Cl; columns 3 and 6, choline; columns 4 and 7, methylamine. The concentrations of the carbon and nitrogen sources are given in Methods. (b) Northern analysis. Twenty micrograms of total RNA was loaded on each lane and probed with ³²P-labelled *FLD1* or *C. boidinii* *ACT1* (actin) DNA. The *C. boidinii* *ACT1* DNA was used as the control for constitutive expression. Each lane number corresponds to the combination of carbon and nitrogen sources listed in (a).

intron (Fig. 1b). The intron sequence contained typical yeast intron sequences, splice junctions (5'-junction, 5'-GTAAGT-3'; 3'-junction, 5'-TAG-3'), and a branch point (5'-TACTAAC-3') (Domdey *et al.*, 1984; Sasnauskas *et al.*, 1992). The *P. pastoris* *FLD1* has been reported to have an intron at the corresponding position (Shen *et al.*, 1998), but *H. polymorpha* FLD has no such intron (Baerends *et al.*, 2002).

The predicted amino acid sequence of FLD from *C. boidinii* showed 80%, 84%, 75%, 80%, and 80% identity to those of FLDs from *P. pastoris* (Shen *et al.*, 1998), *H. polymorpha* (Baerends *et al.*, 2002), *Candida maltosa* (Sasnauskas *et al.*, 1992), and *Saccharomyces cerevisiae* (Wehner *et al.*, 1993), respectively. These belong to a zinc-containing alcohol dehydrogenase family (Sun & Plapp, 1992). The cysteine residues (amino acid positions 47, 109, 112, 115, 123, and 177) and histidine residue (amino acid position 69) previously found to be within the active site (Sasnauskas *et al.*, 1992) are also conserved in FLD from *C. boidinii*.

Fig. 4. Growth of the wild-type and *fld1Δ* strains on various carbon and nitrogen sources, and the formaldehyde concentration in the medium. Cells were grown (a, b and c) in a batch culture of MI basal medium with the carbon and nitrogen sources indicated: (a) methanol medium, (b) glucose+choline medium, (c) glucose+methylamine medium. ●, Growth of the wild-type strain; ○, growth of the *fld1Δ* strain; ▲, formaldehyde concentration with the wild-type strain; △, formaldehyde concentration with the *fld1Δ* strain.

Regulation of *FLD1* expression with various carbon and nitrogen sources

We predicted that *FLD1* expression might be regulated not only by methanol but also by methylamine or choline, whose metabolism also yields formaldehyde.

At first, the regulation of FLD enzyme activity was studied with various combinations of carbon and nitrogen sources for the *C. boidinii* wild-type strain (Fig. 3). Among the carbon sources tested, when NH_4Cl was used as a single nitrogen source, only methanol gave a high level of FLD activity (Fig. 3a, column 1). Glucose- and glycerol-grown cells did not exhibit induced levels of FLD activity (columns 2 and 5). However, when methylamine or choline was used as a single nitrogen source, FLD activity was induced in glucose- or glycerol-grown cells (columns 3, 4, 6 and 7). These results suggest that *FLD1* expression was induced by formaldehyde. Indeed, the addition of formaldehyde (approx. 1 mM) to the glucose/ NH_4Cl medium (column 3) induced FLD activity (column 8).

Next, we conducted Northern analysis of *FLD1* expression using total RNAs extracted from wild-type *C. boidinii* cells grown on each set of carbon and nitrogen sources. As shown in Fig. 3(b), the band intensity paralleled the FLD enzyme activity shown in Fig. 3(a). Therefore, the *FLD1* expression observed was confirmed to be controlled mainly at the mRNA level. These results indicated that the expression of *FLD1* is induced by methanol, formaldehyde, methylamine and choline. Furthermore, the induction of *FLD1* expression is not subject to glucose repression, which is distinct from the case of the AOD or DHAS genes of other methanol metabolism systems (Yurimoto *et al.*, 2000b). A similar pattern of regulation to that of *FLD1* expression was previously confirmed for *FDH1* expression in *C. boidinii* and *FLD1* expression in *P. pastoris* (Sakai *et al.*, 1997; Shen *et al.*, 1998). In addition, the level of

expression under the *FLD1* promoter was comparable to that under the *AOX1* gene promoter in *P. pastoris* (Shen *et al.*, 1998).

Disruption of the *FLD1* gene causes a severe defect in growth on FLD-inducing C and N sources

An *FLD1*-gene disruption vector was constructed and then introduced into the chromosome of *C. boidinii* TK62 (*ura3*) to yield the *fld1Δ* strain. Correct gene disruption was confirmed by genomic Southern analysis with *Hind*III-digested genomic DNA and a ^{32}P -labelled probe. The DNA from the wild-type strain gave a single 2.5 kb band. This band shifted to 6.5 and 3.1 kb for the *fld1Δ* and *fld1Δura3* strains, respectively, as expected for *FLD1* disruption and subsequent deletion of the *URA3* sequence caused by a homologous recombination (Fig. 2). In addition, methanol-induced cells of the *fld1Δ* strain did not exhibit detectable FLD activity (data not shown). These results confirmed that *C. boidinii* contains only one gene encoding FLD.

Next, we compared the growth of the wild-type and *fld1Δ* strains on various carbon sources with NH_4Cl as the nitrogen source. The growth of both strains on glucose or glycerol was similar (data not shown). In contrast, the *fld1Δ* strain could not grow on methanol as the sole carbon source (Fig. 4a). The level of formaldehyde accumulation in the culture medium was determined. With the wild-type strain, formaldehyde accumulated in the medium up to 0.45 mM at 36 h of culture, but then rapidly disappeared. On the other hand, with the *fld1Δ* strain, the formaldehyde level gradually increased up to 0.12 mM and did not decrease until 110 h. These results indicated that formaldehyde consumption was inhibited in the *fld1Δ* strain.

For growth of *C. boidinii* with glucose as the carbon source and choline or methylamine as the nitrogen source, the growth rates of the *fld1Δ* strain ($\mu = 0.046 \text{ h}^{-1}$)

Fig. 5. Growth of the wild-type and *fld1*Δ strains under methanol-limited chemostat culture conditions. Both strains were precultured on glucose-containing medium in a 1.5 l jar fermenter; after the OD₆₁₀ reached 12, feeding of methanol-medium was started at a dilution rate of 0.05 h⁻¹. ●, Growth of the wild-type strain; ○, growth of the *fld1*Δ strain; ▲, formaldehyde concentration with the wild-type strain; △, formaldehyde concentration with the *fld1*Δ strain.

for Fig. 4b and $\mu=0.037$ h⁻¹ for Fig. 4c) were slower than those of the wild-type strain ($\mu=0.17$ h⁻¹ for Fig. 4b and $\mu=0.18$ h⁻¹ for Fig. 4c). In both cases, the formaldehyde level accumulated in the culture medium of the *fld1*Δ strain was higher than that of the wild-type strain.

This growth defect observed for the *fld1*Δ strain was more severe than that observed for the *fdh1*Δ strain in all cases (data not shown) (Sakai *et al.*, 1997). In addition, this growth defect, as well as FLD activity, recovered to levels equivalent to those in the wild-type strain on the introduction of the *FLD1* gene into the *fld1*Δ*ura3* strain (data not shown).

FLD is essential for growth on methanol

The accumulation of formaldehyde could be minimized by the use of methanol-limited chemostat culture conditions with low dilution rates (less than about 0.05 h⁻¹), because the supply of methanol is the rate-limiting factor for methanol metabolism under these conditions. Previously, we showed that the *C. boidinii* *fdh1*Δ strain could grow on methanol as a single carbon source in a methanol-limited chemostat culture, although the growth yield was only one-fourth that of the wild-type strain (Sakai *et al.*, 1997). Therefore, the growth of the wild-type and *fld1*Δ strains was compared under methanol-limited chemostat cultivation conditions (Fig. 5), which were previously applied for analysis of the *fdh1*Δ strain (Sakai *et al.*, 1997). Both the wild-type and *fld1*Δ strains were first grown on glucose medium, and then feeding of methanol medium was started at the dilution rate of 0.05 h⁻¹ to observe the growth at the transition state. As expected, the cell concentration of the wild-type strain became constant after about 60 h (at 3 volume changes of the working volume), suggesting

that the cells had reached methanol-limited chemostat conditions. In contrast, cells of the *fld1*Δ strain were washed out upon feeding with methanol medium. A similar result was obtained with a dilution rate of 0.03 h⁻¹ (data not shown). These results indicate that the *fld1*Δ strain, distinct from the *fdh1*Δ strain, was not able to grow on methanol even under methanol-limited chemostat conditions. We conclude that FLD is essential for growth on methanol and suggest that this enzyme plays critical roles not only in the detoxification of formaldehyde but also in generation of energy in the form of NADH.

DISCUSSION

In this study, we cloned the *FLD1* gene encoding FLD from *C. boidinii* and studied its induction by several carbon and/or nitrogen sources, i.e. methanol, choline and methylamine. The importance of FLD in C₁-metabolic processes was evaluated as the ability of the *fld1*Δ strain to grow on these carbon and nitrogen sources. The observed growth defect clearly demonstrated that FLD is indeed involved in these metabolic processes. We further showed, using a methanol-limited chemostat, that FLD is essential for growth on methanol.

The formaldehyde oxidation pathway comprises two NADH-generating reactions catalysed by two dehydrogenases, i.e. FLD and FDH (Anthony, 1982). The generated NADH molecules had been assumed to be the main source of energy during growth on methanol as a single carbon source. However, the fact that both FLD- and FDH-negative mutant strains of *H. polymorpha* could grow on methanol under methanol-limited chemostat culture conditions raised the possibility that the main function of the formaldehyde oxidation pathway is not energy generation but the detoxification of formaldehyde (Sibirny *et al.*, 1990). Our present and previous studies on FLD and FDH, which involved gene disruption and careful chemostat cultures, clearly showed that there is a difference between the *fld1*Δ and *fdh1*Δ strains in their ability to grow on methanol. The *fdh1*Δ strain could survive and grow on methanol, although the growth yield of this strain was diminished (Sakai *et al.*, 1997). In contrast, the *fld1*Δ strain could not grow on methanol. This difference can be explained by the two physiological functions of the formaldehyde oxidation pathway, i.e. (1) generation of energy and (2) detoxification of formaldehyde. The *fdh1*Δ strain can still generate half of the amount of NADH compared to the wild-type strain through the FLD-catalysed reaction. In contrast, NADH is not expected to be generated in the *fld1*Δ strain, because this strain cannot provide formate, another substrate for NADH generation. This difference in NADH yield clearly explains the difference in growth yield between the *fld1*Δ and *fdh1*Δ strains. Regarding formaldehyde detoxification, formate is at least 10-fold less toxic than formaldehyde (Sakai *et al.*, 1997). Therefore, the *fdh1*Δ strain could survive and grow on methanol when formate accumulated at a

significant level (Sakai *et al.*, 1997). But the high toxicity of formaldehyde will not permit the *fld1Δ* strain to grow on methanol.

Although FLD has two physiological roles, i.e. energy generation and formaldehyde detoxification, we speculate that FLD is more critically involved in growth on methanol through the former rather than the latter function, based on the following observations. (1) The *fld1Δ* strain could survive and grow on choline or methylamine as the nitrogen source, formaldehyde being generated during their metabolism. In these cells, the dissimilation of carbon sources may be the main source of energy generation. Therefore, yeast cells without FLD can tolerate a certain level of formaldehyde. (2) However, the *fld1Δ* strain could not survive under methanol-limited chemostat conditions with very low dilution rates ($<0.05\text{ h}^{-1}$), with which the accumulation of formaldehyde within the cells is expected to be minimized. If FLD only had a formaldehyde detoxification function, the *fld1Δ* strain would grow under these chemostat conditions.

While the formaldehyde oxidation pathway functions mainly as an energy source during growth on methanol, it seems to mainly have a formaldehyde detoxification function in methylamine and choline metabolism. As shown in Fig. 4, a higher level of formaldehyde accumulation is observed in the *fld1Δ* strain than the wild-type strain when cells are grown on glucose as the carbon source and choline or methylamine as the nitrogen source. Since these cells are assumed to generate energy mainly through the glycolytic pathway, the observed growth inhibition may be due to the toxicity of accumulated formaldehyde.

The formaldehyde oxidation pathway is not only found in methylotrophs but also in non-methylotrophs, from lower prokaryotes to higher organisms (Harms *et al.*, 1996). During evolution, possibly, the formaldehyde oxidation pathway was originally used for the detoxification of formaldehyde in many organisms, and thereafter the formaldehyde oxidation pathway in some yeast cells might have acquired an energy-generation function resulting in development of the C_1 pathway, which is now generally found in methylotrophic yeasts.

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REFERENCES

- Anthony, C. (1982). *The Biochemistry of Methylotrophs*. London: Academic Press.
- Baerends, R. J., Sulter, G. J., Jeffries, T. W., Cregg, J. M. & Veenhuis, M. (2002). Molecular characterization of the *Hansenula polymorpha* FLD1 gene encoding formaldehyde dehydrogenase. *Yeast* **19**, 37–42.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Bystrykh, L. V., Aminova, L. R. & Trotsenko, Y. A. (1988). Methanol metabolism in mutants of the methylotrophic yeast *Hansenula polymorpha*. *FEMS Microb Lett* **51**, 89–94.
- Church, G. M. & Gilbert, W. (1984). Genomic sequencing. *Proc Natl Acad Sci USA* **81**, 1991–1995.
- Cryer, D. R., Eccleshal, R. & Murmur, J. (1975). Isolation of yeast DNA. *Methods Cell Biol* **12**, 39–44.
- Davis, R. W., Thomas, M., Cameron, J., John, T. P. S., Scherer, S. & Padgett, R. A. (1980). Rapid DNA isolation for enzymatic and hybridization analysis. *Methods Enzymol* **65**, 404–411.
- Domdey, H., Apostol, B., Lin, R. J., Newman, A., Brody, E. & Abelson, J. (1984). Lariat structures are in vivo intermediates in yeast pre-mRNA splicing. *Cell* **39**, 611–621.
- Feldman, M. Y. (1973). Reactions of nucleic acids and nucleoproteins with formaldehyde. *Prog Nucleic Acids Res Mol Biol* **13**, 1–49.
- Grafstrom, R. C., Fornace, A. J., Jr, Autrup, H., Lechner, J. F. & Harris, C. C. (1983). Formaldehyde damage to DNA and inhibition of DNA repair in human bronchial cells. *Science* **220**, 216–218.
- Harms, N., Ras, J., Reijnders, W. N., van Spanning, R. J. M. & Stouthamer, A. H. (1996). S-Formylglutathione hydrolase of *Paracoccus denitrificans* is homologous to human esterase D: a universal pathway for formaldehyde detoxification? *J Bacteriol* **178**, 6296–6299.
- Horiguchi, H., Yurimoto, H., Kato, N. & Sakai, Y. (2001). Antioxidant system within yeast peroxisome: biochemical and physiological characterization of CbPmp20 in the methylotrophic yeast *Candida boidinii*. *J Biol Chem* **276**, 14279–14288.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**, 416–421.
- Sakai, Y. & Tani, Y. (1992a). Directed mutagenesis in an asporogenous methylotrophic yeast: cloning, sequencing, and one-step gene disruption of the 3-isopropylmalate dehydrogenase gene (*LEU2*) of *Candida boidinii* to derive doubly auxotrophic marker strains. *J Bacteriol* **174**, 5988–5993.
- Sakai, Y. & Tani, Y. (1992b). Cloning and sequencing of the alcohol oxidase-encoding gene (*AOD1*) from the formaldehyde-producing asporogenous methylotrophic yeast, *Candida boidinii* S2. *Gene* **114**, 67–73.
- Sakai, Y., Kazarimoto, T. & Tani, Y. (1991). Transformation system for an asporogenous methylotrophic yeast, *Candida boidinii*: cloning of the orotidine-5'-phosphate decarboxylase gene (*URA3*), isolation of uracil auxotrophic mutants, and use of the mutants for integrative transformation. *J Bacteriol* **173**, 7458–7463.
- Sakai, Y., Goh, T. K. & Tani, Y. (1993). High-frequency transformation of a methylotrophic yeast, *Candida boidinii*, with autonomously replicating plasmids which are also functional in *Saccharomyces cerevisiae*. *J Bacteriol* **175**, 3556–3562.
- Sakai, Y., Saigannji, A., Yurimoto, H., Takabe, K., Saiki, H. & Kato, N. (1996). The absence of Pmp47, a putative yeast peroxisomal transporter, causes a defect in transport and folding of a specific matrix enzyme. *J Cell Biol* **134**, 37–51.
- Sakai, Y., Murdanoto, A. P., Konishi, T., Iwamatsu, A. & Kato, N. (1997). Regulation of the formate dehydrogenase gene, *FDH1*, in the methylotrophic yeast *Candida boidinii* and growth characteristics of an *FDH1*-disrupted strain on methanol, methylamine, and choline. *J Bacteriol* **179**, 4480–4485.
- Sakai, Y., Nakagawa, T., Shimase, M. & Kato, N. (1998). Regulation and the physiological role of the *DAS1* gene encoding

dihydroxyacetone synthase in the methylotrophic yeast *Candida boidinii*. *J Bacteriol* **180**, 5885–5890.

Sasnauskas, K., Jomantiene, R., Januska, A., Lebediene, E., Lebedys, J. & Janulaitis, A. (1992). Cloning and sequencing analysis of a *Candida maltosa* gene which confers resistance to formaldehyde in *Saccharomyces cerevisiae*. *Gene* **122**, 207–211.

Schutte, H., Flossdorf, J., Sahm, H. & Kula, M.-R. (1976). Purification and properties of formaldehyde dehydrogenase and formate dehydrogenase from *Candida boidinii*. *Eur J Biochem* **62**, 151–160.

Shen, S., Sulter, G., Jeffries, T. W. & Cregg, J. M. (1998). A strong nitrogen source-regulated promoter for controlled expression of foreign genes in the yeast *Pichia pastoris*. *Gene* **216**, 93–102.

Sibirny, A. A., Ubiyvovk, V. M., Gonchar, M. V., Titorenko, V. I., Voronovsky, A. Y., Kapultsevich, Y. G. & Bliznik, K. M. (1990). Reactions of direct formaldehyde oxidation to CO₂ are non-essential for energy supply of yeast methylotrophic growth. *Arch Microbiol* **154**, 566–575.

Sun, H. W. & Plapp, B. V. (1992). Progressive sequence alignment and molecular evolution of the Zn-containing alcohol dehydrogenase family. *J Mol Evol* **34**, 522–535.

Tani, Y., Sakai, Y. & Yamada, H. (1985). Production of formaldehyde by a mutant of methanol yeast, *Candida boidinii* S2. *J Ferment Technol* **63**, 443–449.

Veenhuis, M., van Dijken, J. P. & Harder, W. (1983). The significance of peroxisomes in the metabolism of one-carbon compounds in yeasts. *Adv Microb Physiol* **24**, 1–82.

Wehner, E. P., Rao, E. & Brendel, M. (1993). Molecular structure and genetic regulation of *SFA*, a gene responsible for resistance to formaldehyde in *Saccharomyces cerevisiae*, and characterization of its protein product. *Mol Gen Genet* **237**, 351–358.

Yurimoto, H., Hasegawa, T., Sakai, Y. & Kato, N. (2000a). Physiological role of the D-amino acid oxidase gene, *DAO1*, in carbon and nitrogen metabolism in the methylotrophic yeast *Candida boidinii*. *Yeast* **16**, 1217–1227.

Yurimoto, H., Komeda, T., Lim, C. R., Nakagawa, T., Kondo, K., Kato, N. & Sakai, Y. (2000b). Regulation and evaluation of five methanol-inducible promoters in the methylotrophic yeast *Candida boidinii*. *Biochim Biophys Acta* **1493**, 56–63.

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