INTRODUCTION

Methylotrophic yeasts, which are capable of utilizing methanol as the sole carbon and energy source, are widely used for the production of heterologous proteins and also as model organisms for studies on peroxisome biogenesis and degradation (Gellissen, 2000; Subramani, 1998). Methanol metabolism starts with the oxidation of methanol to formaldehyde, a reaction catalysed by alcohol oxidase (AOD, EC 1.1.3.13) (Fig. 1) (Yurimoto et al., 2002). Formaldehyde is the central intermediate of methylotrophic metabolism, being situated at the branching point of the assimilation and dissimilation pathways. A portion of formaldehyde is fixed into xylulose 5-phosphate by dihydroxyacetone synthase (EC 2.2.1.3) and then enters the dissimilation pathways. A portion of formaldehyde spontaneously and non-enzymically reacts with the reduced form of glutathione (GSH) to form S-hydroxymethylglutathione, and then is successively oxidized to carbon dioxide through the glutathione-dependent formaldehyde oxidation pathway, which involves NAD$^+$- and glutathione-dependent formaldehyde dehydrogenase (FDH, EC 1.2.1.1), S-formylglutathione hydrolase (FGH, EC 3.1.2.12), and NAD$^+$-dependent formate dehydrogenase (FDH, EC 1.2.1.2). These sequential reactions are considered to participate in the energy formation and detoxification of formaldehyde in C$_1$ metabolism of methylotrophic yeasts (Lee et al., 2002). When a methylotrophic yeast is grown on methanol as the sole carbon source, methanol-limited chemostat conditions with low dilution rates ($D<0.05$ h$^{-1}$), conditions under which a strain with disruption of the gene for formaldehyde dehydrogenase (another enzyme involved in the formaldehyde oxidation pathway) could not survive. These results suggested that FGH is not essential but necessary for optimal growth on methanol. This is believed to be the first report of detailed analyses of the FGH1 gene in a methylotrophic yeast strain.

Abbreviations: AOD, alcohol oxidase; FDH, formate dehydrogenase; FGH, S-formylglutathione hydrolase; FLD, glutathione-dependent formaldehyde dehydrogenase; GFP, green fluorescent protein; GSH, reduced form of glutathione; GS-GS, oxidized form of glutathione; PTS, peroxisome-targeting signal.

The GenBank accession number for the sequence reported in this paper is AB104827.

Physiological role of S-formylglutathione hydrolase in C$_1$ metabolism of the methylotrophic yeast Candida boidinii

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The methylotrophic yeast Candida boidinii exhibits S-formylglutathione hydrolase activity (FGH, EC 3.1.2.12), which is involved in the glutathione-dependent formaldehyde oxidation pathway during growth on methanol as the sole carbon source. The structural gene, FGH1, was cloned from C. boidinii, and its predicted amino acid sequence showed more than 60% similarity to those of FGHs from Paracoccus denitrificans and Saccharomyces cerevisiae, and human esterase D. FGH from C. boidinii contained a C-terminal tripeptide, SKL, which is a type I peroxisome-targeting signal, and a bimodal distribution of FGH between peroxisomes and the cytosol was demonstrated. The FGH1 gene was disrupted in the C. boidinii genome by one-step gene disruption. The fgh1Δ strain was still able to grow on methanol as a carbon source under methanol-limited chemostat conditions with low dilution rates ($D<0.05$ h$^{-1}$), conditions under which a strain with disruption of the gene for formaldehyde dehydrogenase (another enzyme involved in the formaldehyde oxidation pathway) could not survive. These results suggested that FGH is not essential but necessary for optimal growth on methanol. This is believed to be the first report of detailed analyses of the FGH1 gene in a methylotrophic yeast strain.

Abbreviations: AOD, alcohol oxidase; FDH, formate dehydrogenase; FGH, S-formylglutathione hydrolase; FLD, glutathione-dependent formaldehyde dehydrogenase; GFP, green fluorescent protein; GSH, reduced form of glutathione; GS-GS, oxidized form of glutathione; PTS, peroxisome-targeting signal.

The GenBank accession number for the sequence reported in this paper is AB104827.
The P. denitrificans fghA mutant was unable to grow on methanol as a carbon source, indicating that FGH is essential for methylotrophic growth (Harms et al., 1996). Although S. cerevisiae could not grow on C₁ compounds, the YJL068C-null mutant grew more slowly in the presence of formaldehyde than the wild-type strain (Degrassi et al., 1999). Recent genomic sequence analyses have also revealed the existence of putative FGH-encoding genes in a variety of organisms, leading to the proposal that FGH and the glutathione-dependent formaldehyde oxidation pathway are distributed throughout eukaryotes and prokaryotes (Harms et al., 1996). These reports suggest that the main physiological role of FGH is the detoxification of formaldehyde.

We are studying the physiological role of the glutathione-dependent formaldehyde oxidation pathway using gene-disrupted strains of the methylotrophic yeast Candida boidinii in combination with the methanol-limited chemostat culture technique (Lee et al., 2002; Sakai et al., 1997). Our previous studies demonstrated that FLD, but not FDH, was essential for growth on methanol, although FDH was necessary for optimal growth on methanol. FGH has been purified to homogeneity from two strains of C. boidinii (Kato et al., 1980; Neben et al., 1980). FGH may also play an important role in the regeneration of GSH within cells, since GSH is involved in the detoxification of reactive oxygen species during methanol metabolism (Horiguchi et al., 2001a; Ubiyvok et al., 2002). Distinct from the other two NADH-producing dehydrogenases, the reaction catalysed by FGH, i.e. hydrolysis of S-formylglutathione, might occur non-enzymically or be catalysed by other hydrolytic enzymes in the cells. However, when compared with those of FLD and FDH, our knowledge of the physiological role of FGH in yeast C₁ metabolism is very limited, because there have been no reports on gene cloning or mutant analysis of FGH from any methylotrophic yeast strains. We believe this report to be the first to describe (i) the characterization of an FGH-encoding gene from a methylotrophic yeast, (ii) gene-disruption analyses in combination with the methanol-limited chemostat culture technique; and (iii) the subcellular localization of FGH in C. boidinii. The results obtained are discussed from the viewpoint of the physiological roles of FGH and glutathione in yeast C₁ metabolism.

**METHODS**

**Yeast strains, media, and cultivation.** C. boidinii S2 (Tani et al., 1985) was the origin of the chromosomal DNA and was used as the wild-type strain. C. boidinii TK62 (ura3) (Sakai et al., 1991) was used as the host for transformation. Yeast cultures were grown on the synthetic MI medium described previously (Sakai et al., 1991), with the carbon and nitrogen sources as follows: 1-5 % (v/v) methanol, 2 % (w/v) glucose, 0-76 % (w/v) NH₄Cl, 0-5 % (w/v) methylamine hydrochloride and 0-5 % (w/v) choline chloride. The initial pH of the media was adjusted to 6-0. Cultivation was performed under aerobic conditions at 28 °C with reciprocal shaking, and growth was monitored by measuring the OD₆10. The methanol-limited chemostat culture was performed as described previously (Lee et al., 2002).

**DNA and RNA methods.** Yeast DNA was isolated by the method of Cryer et al. (1975) or Davis et al. (1980). Southern analysis was performed as described previously (Yurimoto et al., 2000a). Transformation of C. boidinii was performed by the modified lithium acetate method, as described previously (Sakai et al., 1993). pBluescript II SK+ was from Stratagene. DNA was sequenced with a 7-deaza sequencing kit from Amersham Biosciences and a DNA sequencer model DSQ-2000L from Shimadzu. Total RNA extraction and Northern analysis were performed as described previously (Lee et al., 2002).

**Enzyme assays and protein methods.** We determined FGH activity by measuring the esterase activity towards p-nitrophenyl acetate as a substrate. The activity was determined by monitoring the release of p-nitrophenol photometrically at 420 nm (Degrassi et al., 1999). One enzyme unit was taken as the amount of enzyme releasing 1 μmol p-nitrophenol min⁻¹ at pH 6-0 and 30 °C under the above assay conditions. Protein was determined with a protein assay kit (Bio-Rad) with bovine serum albumin as the standard.

Standard 9 % Laemmli gels (Laemmli, 1970), with the separating gels at pH 9-2, were used. Immunoblotting was performed by the method of Towbin et al. (1979) using an ECL detection kit (Amersham).
The anti-GFP antibody was kindly provided by Dr M. Fransen (Katholieke Universiteit, Leuven, Belgium). Subcellular fractionation was performed as described previously (Horiguchi et al., 2001). The enzyme activities of AOD and cytochrome c oxidase were assayed as described previously (Sakai et al., 1996).

**Cloning of the C. boidinii FGH1 gene.** Based on the amino acid sequences of highly conserved regions in several FGH-encoding genes (VFYLSGLTC and DHSYYFVS in *S. cerevisiae YJL068C*) and the preferred codon usage in *C. boidinii*, two mixed primers, primer 1 and primer 2, were designed (Table 1). Using these two primers and *C. boidinii* genomic DNA as a template, a 0.7 kb fragment was specifically amplified. Nucleotide sequence analysis showed that the amino acid sequence deduced from the nucleotide sequence of the 0.7 kb fragment was highly similar to those of other FGHs. Therefore, the resultant 0.7 kb fragment was gel-purified and used as a probe for hybridization experiments.

Genomic Southern analysis of the genomic DNA from *C. boidinii* S2 digested with nine different restriction enzymes showed that a 3.0 kb *Eco*RV fragment hybridized to the probe. *Eco*RV-digested chromosomal DNA corresponding to a size of about 3.0 kb was ligated into the *Eco*RV site of pBluescript II SK+, and then transformed into *Escherichia coli* DH5α. Colony hybridization was performed as described previously (Yurimoto et al., 2000). Clones that showed strong signals were picked up from the original plates and used for further studies (pFGH).

**Construction of an FGH1 gene disruption cassette and one-step gene disruption.** pFGH was digested with *Nde I* and *Nco I* to remove an approximately 143 bp fragment of the partial coding sequence of FGH1. The remaining linearized plasmid and the 4.6 kb *Sac I–Xho I* fragment of *C. boidinii* URA3 DNA from pSPR (Sakai & Tani, 1992) were blunt-ended with T4 DNA polymerase and then ligated, yielding FGH1 disruption vector pFGHD. pFGHD had *C. boidinii* URA3 DNA as a selectable marker and truncated FGH1-flanking sequences. pFGHD was digested with *Sac I* and *Xho I*, and then used for the transformation of *C. boidinii* strain TK62. The FGH1-disrupted (fgh1Δ) strain was caused to revert to uracil auxotrophy after 5-fluoroorotic acid selection, yielding the fgh1Δura3 strain, by our previously described procedure (Sakai & Tani, 1992). The gene disruption and loss of URA3 were confirmed by genomic Southern

**Table 1. Oligonucleotide primers used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer 1</td>
<td>TWTTTAYTYTTWCCGWTNNACNTG</td>
</tr>
<tr>
<td>Primer 2</td>
<td>WGGWACAAARTARTAWGWRGATC</td>
</tr>
<tr>
<td>GFPATG</td>
<td>ACCGCGTACATGGAAGAAAGAAGCTTT</td>
</tr>
<tr>
<td>GFPFGH1</td>
<td>GATGAACTATAACAAGCCGAGTTCATTAAATTGAATCTGAGATTG</td>
</tr>
<tr>
<td>FGH1GFP</td>
<td>CAATCTCATTCAATTTAATGAACCTCCGGACTTGTATAGTTCACTC</td>
</tr>
<tr>
<td>FGH1PstI3'</td>
<td>AACTGCACTTTAATTTTGTATGAACCTAAAT</td>
</tr>
<tr>
<td>DSKLPstI</td>
<td>AACTGCACTTTAATTTTGTATGAACCTAAAT</td>
</tr>
</tbody>
</table>

![Fig. 2. Physical map of the cloned FGH1 gene and one-step disruption of the FGH1 gene in *C. boidinii*. (a) Restriction map of the cloned fragment and gene-disruption strategy. The shaded boxes at both ends of URA3 show repeated sequences for homologous recombination to remove the URA3 gene after gene disruption. (b) Genomic Southern analysis of XbaI-digested total DNAs (3 μg of each) from host strain TK62 (lane 1), the fgh1Δ strain (lane 2), and the fgh1Δura3 strain (lane 3) probed with the 1.0 kb EcoRV–Nde I fragment harbouring the 5′-flanking region of FGH1.](http://mic.sgmjournals.org)
analysis using XbaI-digested genomic DNA from each transformant with the 1-0 kb EcoRV–Ndel fragment harbouring the 5′-flanking region of FGH as the probe (Fig. 2b).

**Construction of strains expressing green fluorescent protein (GFP)-fused FGH1.** A PCR technique was used to construct GFP-fusion proteins and their expression vectors. To construct pGFP–FGH1, two rounds of PCR were performed using primers GFPATG and GFPFGH1 (Table 1) with pGFP–CI (CLONTECH) as the template, and primers FHG1 GFP and FGHHPTsp13′ (Table 1) with C. boidinii FGH1 DNA as the template, respectively. Next, the two amplified fragments were gel-purified and used as PCR templates using primers GFPATG and FGHHPTsp13′. The amplified fragment was gel-purified, digested with SalI and PstI, and then introduced into C. boidinii expression vector pACT1 (Sakai et al., 1998). pACT1 harboured the C. boidinii ACT1 promoter and terminator sequences with a unique SalI–PstI site to insert coding sequences for expression. pGFP–FGH1ASKL was constructed in exactly the same manner, except that primer DSKLPstI was used instead of FGHHPTsp13′ (Table 1). These plasmids constructs were linearized with XbaI and then introduced into the fgh1ura3 strain.

**RESULTS**

**Molecular cloning of C. boidinii FGH1 and its primary structure**

Synthetic mixed primers, primer 1 and primer 2, were designed based on amino acid sequences that are highly conserved regions in FGH-encoding genes. The PCR reaction with primer 1 and primer 2, and C. boidinii genomic DNA, yielded an approximately 0.7 kb fragment. The DNA sequence of the amplified fragment could code for a protein showing high similarity to amino acid sequences of FGHs from P. denitrificans and S. cerevisiae. On genomic Southern analysis with the 0.7 kb PCR amplified fragment as the probe, a single 3-0 kb band was observed with EcoRV-digested C. boidinii genomic DNA. The corresponding DNA of this size was gel-purified and a gene library was constructed on pBluescript II SK+. Colony hybridization selection gave five independent positive clones exhibiting identical physical maps (Fig. 2a).

Only one ORF, of 900 bp, was found. There was no evidence of the presence of an intron in the ORF. From the deduced amino acid sequence, the molecular mass of the protein was calculated to be 33 655 Da (excluding the first Met residue), which corresponded to 31 kDa for the purified enzyme (Kato et al., 1980).

The deduced amino acid sequence of the identified ORF showed 75%, 62% and 66% similarity to those of S. cerevisiae, P. denitrificans and human esterase D, respectively. In the central section, Ser156 of C. boidinii is in the consensus sequence GXSXG around the catalytic serine, typical of all lipases and esterases (Fernandez et al., 2000). Near the C-terminus, conserved His273 is found, which is predicted to be the catalytic histidine residue (Cygler et al., 1993). The alignment suggested that Asp238 might be the catalytic residue, since this residue is conserved in other FGHs (Derewenda, 1994). In addition, the extreme C-terminal sequence of FGH from C. boidinii is -SKL, which is a typical peroxisome-targeting signal 1 (PTS1) found in peroxisomal enzymes (Elgersma et al., 1996; Nishikawa et al., 2000). Subcellular localization of FGH and the role of C-terminal -SKL are described below. From the results of these and following gene disruption analyses, we concluded that this ORF corresponds to the gene for FGH in C. boidinii, FGH1.

**Regulation of FGH1 expression**

Previously, we reported that two other genes involved in the glutathione-dependent formaldehyde oxidation pathway, FLD1 and FDH1, were induced by methanol, methylamine and choline. Since the latter two compounds yield formaldehyde when they are used as nitrogen sources, formaldehyde induced the FLD1 and FGH1 expression (Lee et al., 2002; Sakai et al., 1997). The induction of FLD1 and FDH1 expression was not subject to glucose repression, this feature being distinct from that of the AOD-encoding gene (Yurimoto et al., 2000b).

The regulation of FGH1 expression by carbon and nitrogen sources was assessed by (i) Northern analysis with FGH1 DNA as a probe or (ii) esterase enzyme assaying with p-nitrophenyl acetate as a substrate (Fig. 3). When the
wild-type strain was grown on glucose as the sole carbon source, no FGH1 mRNA was detected (Fig. 3a). However, a high amount of FGH1 mRNA was detected when cells were grown on methanol. The level of esterase activity in glucose-grown cells was lower than that in methanol-grown cells (Fig. 3b). Judging from these Northern analyses and the fact that basal levels of esterase activities were detected in the fgh1Δ strain (Fig. 3b), the esterase activity of glucose-grown cells was due to some esterase activity other than that of FGH, and the esterase activity induced by methanol could be ascribed to FGH activity. Similarly, induced levels of FGH1 mRNA and esterase activity were observed when cells were grown on methylene or choline as the sole nitrogen source. Judging from these results, the activity of FGH is induced by both methanol and formaldehyde, and is not subject to glucose repression, as was previously observed for FLD and FDH (Lee et al., 2002; Sakai et al., 1997). FGH1 expression was confirmed to be controlled mainly at the mRNA level.

Disruption of FGH1, and its effect on growth on methanol, methylamine and choline under batch culture conditions

An FGH1-disruption vector was constructed and then introduced into the chromosome of C. boidinii TK62 to yield the fgh1Δ strain. Since the integrated plasmid had tandem repeated sequences, the URA3 gene of the fgh1Δ strain was expected to excise from the chromosome through site-specific recombination at these repeated sequences at a high frequency. The fgh1Δura3 strain was isolated by its resistance to 5-fluoroorotic acid, as described previously (Sakai & Tani, 1992). The disruption of FGH1 and excision of URA3 were confirmed by genomic Southern analysis (Fig. 2b). The DNA from the wild-type strain gave a single band corresponding to 5·0 kb; this band shifted to 9·4 and 6·0 kb for the fgh1Δ and fgh1Δura3 strains, respectively, as expected for disruption of the FGH1 gene and excision of the URA3 sequence caused by homologous recombination, respectively.

We compared the growth of the wild-type and fgh1Δ strains on methanol (as a carbon source), and methylamine and choline (as nitrogen sources) under batch culture conditions. The growth of both strains on glucose or glycerol as the sole carbon source was similar (data not shown). The fgh1Δ strain could not grow on methanol as the sole carbon source (Fig. 4a). When methylamine or choline was used as the sole nitrogen source, both the growth rate and growth yield of the fgh1Δ strain were lower than those of the wild-type strain (Fig. 4b, c). From these results, FGH was proved to play a significant role in yeast C1 metabolism.

The fgh1Δ strain can grow on methanol under methanol-limited chemostat conditions

Previously, we applied methanol-limited chemostat culture conditions to minimize the formaldehyde level in growing cells. Under these conditions, while the fld1Δ strain was washed out from the culture (Lee et al., 2002), the fdh1Δ strain could survive and grow on methanol as the sole carbon source. The growth yield of the fgh1Δ strain was one-fourth of that of the wild-type strain (Sakai et al., 1997). In the present study, we compared the growth of the wild-type and fgh1Δ strains under the previously described chemostat culture conditions (Fig. 4d). The cell concentration of the wild-type strain became constant about 60 h after the initiation of feeding the medium (at 3 volume changes of the working volume). The cell concentration of the fgh1Δ strain also became constant, after about 80 h, and the growth yield of this strain was approximately one-tenth that of the wild-type strain. The present results indicate that the fgh1Δ strain, distinct from the fld1Δ strain, was able to

![Graphs showing growth on different conditions](http://mic.sgmjournals.org)
grow on methanol under methanol-limited chemostat conditions. We conclude that FGH is not essential but is important for optimal growth on methanol.

**Subcellular localization of FGH in *C. boidinii***

So far it has been thought that glutathione-dependent formaldehyde oxidation occurs exclusively in the cytosol, since both FLD and FGH have been shown to be cytosolic. Unexpectedly, FGH from *C. boidinii* has a C-terminal SKL tripeptide, which is a typical PTS1 sequence. Previously, we showed that a peroxisomal acetylspermidine oxidase also had a C-terminal SKL tripeptide in *C. boidinii* (Nishikawa et al., 2000). Next, we studied the subcellular localization of FGH in *C. boidinii*, and examined the physiological significance of the -SKL tripeptide.

Full-length FGH or C-terminal SKL-truncated FGH was tagged with GFP at the N-terminus, integrated into the *ura3* locus of the *fgh1Δura3* strain to form the GFP–FGH1 and GFP–FGH1ΔSKL strains, respectively, and then expressed under the constitutive *ACT1* promoter. Both strains could complement the growth defect of the *fgh1Δ* strain on methanol (Fig. 4a). Judging from these results, GFP-tagging to FGH did not affect its function, and the C-terminal SKL tripeptide is not essential for its enzymic function nor its physiological function.

Cells of the GFP–FGH1 and GFP–FGH1ΔSKL strains were grown on methanol medium and then observed by fluorescence microscopy. The GFP–AKL strain (Sakai et al., 1998) showed intrinsic green fluorescent punctate structures and the wild-type strain did not show any fluorescence (Fig. 5a). The GFP–FGH strain exhibited not
only cytosolic fluorescence but also punctuate fluorescence, while the GFP–FGH1ΔSKL strain exhibited only cytosolic fluorescence (Fig. 5a). The subcellular localization of these GFP–FGH fusion proteins was further examined by subjecting the GFP–FGH1 and GFP–FGH1ΔSKL strains to differential centrifugation, which separated the intracellular components into a cytosolic supernatant (S) and organelle pellet (P), which mainly contained peroxisomes and mitochondria (Fig. 5b). The activity of AOD, a marker enzyme of peroxisomes, amounted to up to 98% in the pellet fractions of both strains. The GFP proteins were detected in both the supernatant and pellet fractions in about equal amounts for the GFP–FGH1 cells as well as the GFP–AKL cells. Furthermore the GFP–FGH fusion protein in the pellet fraction was found to be co-localized with AOD on Nycodenz-gradient ultracentrifugation (Fig. 5c). These results indicated a bimodal distribution of GFP–FGH between the cytosol and peroxisomes. In contrast, most GFP–FGHASKL proteins were only detected in the supernatant fraction of the GFP–FGH1ΔSKL cells (Fig. 5b).

**DISCUSSION**

In this study, we constructed and characterized an FGH1-knockout strain of the methylotrophic yeast *C. boidinii* to reveal its physiological function in yeast C1 metabolism. The reaction catalysed by FGH, i.e. the formation of formate and glutathione, is speculated to have two important functions in yeast C1 metabolism: as the reaction that supplies GSH to the intracellular pool, and as a component of the formaldehyde oxidation pathway.

Recently, we found that a physiological level of GSH exists within peroxisomes of *C. boidinii* (Horiguchi *et al.*, 2001a). GSH in peroxisomes is required for the glutathione peroxidase activity of CbPmp20, which detoxifies reactive oxygen species within peroxisomes (Horiguchi *et al.*, 2001a). On the other hand, formaldehyde non-enzymically reacts with GSH within peroxisomes to form S-hydroxymethylglutathione. Since FLD is not present in peroxisomes, the S-hydroxymethylglutathione formed must be exported to the cytoplasm. Also, since glutathione reductase activity was not detected in the peroxisome fraction (Horiguchi *et al.*, 2001a), the oxidized form of glutathione (GS-SG) generated by CbPmp20 within peroxisomes must be exported for regeneration of GSH by glutathione reductase. Therefore, there may be some transport system in peroxisomal membrane for importing and exporting glutathione and its related compounds, at least for GSH, GS-SG and S-hydroxymethylglutathione.

Sequence analysis of FGH1 revealed that FGH has a C-terminal tripeptide SKL, which is the consensus sequence for a peroxisomal-targeting signal (PTS1) (Elgersma *et al.*, 1996; Nishikawa *et al.*, 2000). By means of subcellular fractionation experiments and observation of GFP-tagged FGHs by fluorescence microscopy, FGH has been shown to exhibit a bimodal distribution between the cytosol and peroxisomes. A similar bimodal distribution was observed for *C. boidinii* catalase, which was due to the low level of interaction between the C-terminal NKF tripeptide and a PTS1 receptor, Pex5p (Horiguchi *et al.*, 2001b). In yeast C1 metabolism, the oxidation of methanol to formaldehyde by AOD and subsequent assimilation by dihydroxyacetone synthase occur in peroxisomes. In contrast, the formaldehyde oxidation pathway has been thought to exist exclusively in the cytosol because two other enzymes, FLD and FDH, are localized in the cytosol (Yurimoto *et al.*, 2002). One possible explanation for the peroxisomal existence of FGH is as follows: a portion of S-formylglutathione, a product of the FLD-catalysed reaction and a substrate for FGH, is transferred from the cytosol to peroxisomes by some transport system mentioned above, and FGH may release GSH within peroxisomes. It might be interesting to determine whether FGHs from other methylotrophic yeast strains are also present in peroxisomes, although *Pichia pastoris* FGH did not have a putative PTS1 at its C-terminus (unpublished results).

We performed methanol-limited chemostat culture experiments of the knockout strains to study the formaldehyde oxidation pathway enzymes. The accumulation of formaldehyde could be minimized by the use of methanol-limited chemostat 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. Indeed, the fgh1Δ strain, which exhibited only weak growth in a batch culture, could grow on methanol under chemostat conditions with a growth yield of one-fourth that of the wild-type strain (0.55 versus 2.3 g dry cell weight g⁻¹) (Sakai *et al.*, 1997). However, the fld1Δ strain was unable to grow on methanol even under the same chemostat conditions (Lee *et al.*, 2002). Our present results showed that the fgh1Δ strain could grow on methanol as a carbon source and was never washed out from a chemostat culture, although the growth yield of the fld1Δ strain (0.2 g dry cell weight g⁻¹) was only 10% that of the wild-type strain.

We speculate that these differences in the growth yield among the knockout strains reflect the differences in the contribution of each enzyme to C1 metabolism. The formaldehyde oxidation pathway has two physiological functions, i.e. generation of energy and detoxification of formaldehyde. The difference of growth yield on methanol-limited chemostat conditions among the knockout strains can be explained by the aspect of generation of energy. The wild-type strain could yield 2 mol NADH per 1 mol dissimilated formaldehyde, through the formaldehyde oxidation pathway. While both the fgh1Δ and fld1Δ strains could yield 1 mol NADH per 1 mol dissimilated formaldehyde, the fld1Δ strain could not yield NADH at all. These results suggest that (1 mol NADH)/(1 mol dissimilated formaldehyde) is sufficient to support growth on methanol and that the NADH molecules generated through the glutathione-dependent formaldehyde oxidation pathway.
are the main source for energy generation. However, the toxicity of formaldehyde must also be considered. When methylamine or choline was used as the sole nitrogen source, the fgh1Δ strain showed weaker growth than the wild-type strain (Fig. 4b, c). In these conditions, the lower growth rate and growth yield in the fgh1Δ strain were assumed to be caused by the toxicity of formaldehyde, since dissimilation of carbon sources might be the main source of energy. The growth defect of the fgh1Δ strain on methanol may also be caused by the toxicity of formaldehyde.

While the fgh1Δ and fdh1Δ strains were calculated to yield the same amount of NADH, the growth yield of the fgh1Δ strain was less than half that of the fdh1Δ strain. In the fgh1Δ strain, GSH is not released from S-formylglutathione, which would lead to further inefficiency as to formaldehyde oxidation and energy generation. In the wild-type cells as well as those of the fdh1Δ strain, GSH released from S-formylglutathione would be transported to peroxisomes, where GSH could react with formaldehyde to enter into another cycle of the formaldehyde oxidation pathway or could be used as a reducing agent for CbPmp20 antioxidant activity. In fact, the fgh1Δ cells had a lower concentration of GSH [0-125 mmol (mg protein)⁻¹] than the fdh1Δ cells [0-309 mmol (mg protein)⁻¹]. Therefore, besides being a component of the formaldehyde oxidation pathway, FGH seems to play an important role in the regeneration of GSH.

The glutathione-dependent formaldehyde oxidation pathway is found not only in methylo trophs but also in non-methylo trophs (Harms et al., 1996). In general, this pathway is considered to be used for the detoxification of formaldehyde. However, during the evolution of methylo trophs, this pathway might have acquired an energy-generation function as well as that of regulation of the glutathione level in cells.

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