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Peroxisomal Fba2p and Tal2p complementally function in the rearrangement pathway for xylulose 5-phosphate in the methylotrophic yeast *Pichia pastoris*

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In this work, we analyzed several genes participating in the rearrangement pathway for xylulose 5-phosphate (Xu5P) in the methylotrophic yeast *Pichia pastoris* (syn. *Komagataella phaffii*). *P. pastoris* has two set of genes for fructose-1,6-bisphosphate aldolase (*FBA1* and *FBA2*) and transaldolase (*TAL1* and *TAL2*), although there are single-copy genes for fructose-1,6-bisphosphatase (*FBP1*) and transketolase (*TKL1*), respectively. Expressions of *FBP1* and *TAL2* were upregulated by non-fermentative carbon sources, especially methanol was the best inducer for them, and *FBA2* was induced only by methanol. On the other hand, *FBA1, TAL1* and *TKL1* showed constitutive expression. Strain *fbp1*Δ showed severe growth defect on methanol and non-fermentable carbon sources, and growth rate of strain *fba2*Δ in methanol medium was slightly decreased. Moreover, Fba2p and Tal2p possessed peroxisome targeting signal type 1 (PTS1), and EGFP-Fba2p and EGFP-Tal2p were found to be localized in peroxisomes. From these findings, it was suggested that Fba2p, Fbp1p and Tal2p participate in the rearrangement pathway for Xu5P in peroxisomes, and that the altered Calvin cycle and non-oxidative pentose phosphate pathway involving Tal2p function in a complementary manner.

[**Key words:** Fructose-1,6-bisphosphate aldolase; Fructose-1,6-bisphosphatase; Transaldolase; Xylulose 5-phosphate; Methanol metabolism; *Pichia pastoris*]

Methylotrophic yeast is able to utilize methanol as a sole carbon and energy source. Some of the yeast strains, *Pichia pastoris* (syn. *Komagataella phaffii*), *Hansenula polymorpha* (syn. *Ogataea polymorpha*), *Candida boidinii*, and *Pichia methanolica* (syn. *O. methanolica*), have been used as heterologous gene-expression systems using strong methanol-inducible promoters derived from genes encoding the alcohol oxidase (AOX) and other methanol-metabolic enzymes (1–4).

In methanol metabolism of the methylotrophic yeast, the first step is methanol oxidation by AOX in peroxisomes, generating formaldehyde. After the AOX-catalyzed reaction, formaldehyde is fixed to D-xylulose 5-phosphate (Xu5P) as a C_1-acceptor molecule catalyzed by dihydroxyacetone synthase (DAS) in peroxisomes, and then the carbon molecule from formaldehyde is lead to assimilatory pathway of methanol metabolism (Fig. 1). Therefore, Xu5P is one of the indispensable intermediates in the methanol metabolism, and the yeast cells have to provide sufficient amounts of Xu5P to the DAS-catalyzed reaction during methylotrophic growth (5).

Until now, details of the pathway for the supply of Xu5P in methanol metabolism has not been known clearly, and it has been assumed that Xu5P is synthesized at the rearrangement pathway, which includes two stages; the first stage is supplying pathway of D-fructose 6-phosphate (F6P), and the second is non-oxidative pentose phosphate pathway (4,5). In the first stage, dihydroxyacetone kinase (DAK) catalyzes the ATP-dependent phosphorylation of dihydroxyacetone in cytosol (6), and the following reactions are catalyzed by parts of gluconeogenesis, D-fructose-1,6-bisphosphate aldolase (FBA) and D-fructose-1,6-bisphosphatase (FBP), which supplies F6P from non-fermentative carbon sources. At the second stage, it seems that F6P is converted into Xu5P and D-ribose 5-phoshate (R5P) by transketolase (TKL) and transaldolase (TAL), which are member of the non-oxidative pentose phosphate pathway (4,5).

Recently, Ruβmayer et al. (7) proposed metabolic map for the rearrangement pathway for Xu5P in methanol metabolism using data of transcriptomics, proteomics, and metabolomics analyses (Fig. 1A). In their work, it was reported that (i) *P. pastoris* had duplicate methanol inducible enzyme set for the rearrangement pathway, e.g., FBAs and TALs, (ii) induction and the gene expression of the second isozymes, Fba2p and Tal2p, were upregulated by methanol, and (iii) the rearrangement pathway resembled the principle of the Calvin cycle using sedoheptulose 1,7-bisphosphate (S1,7BP) as intermediate. Moreover, they suggested that the whole rearrangement pathway for Xu5P was localized in peroxisomes, because (i) all enzymes participating in the rearrangement pathway were localized in peroxisomes and (ii) DAS also acts as TKL in peroxisomes, since DAS has classical TKL activity (8,9). However, the rearrangement pathway of Xu5P was proposed based on several omics data sets, thus there has been no analysis data about functions of individual enzymes/genes in the methylotrophic yeast cell.

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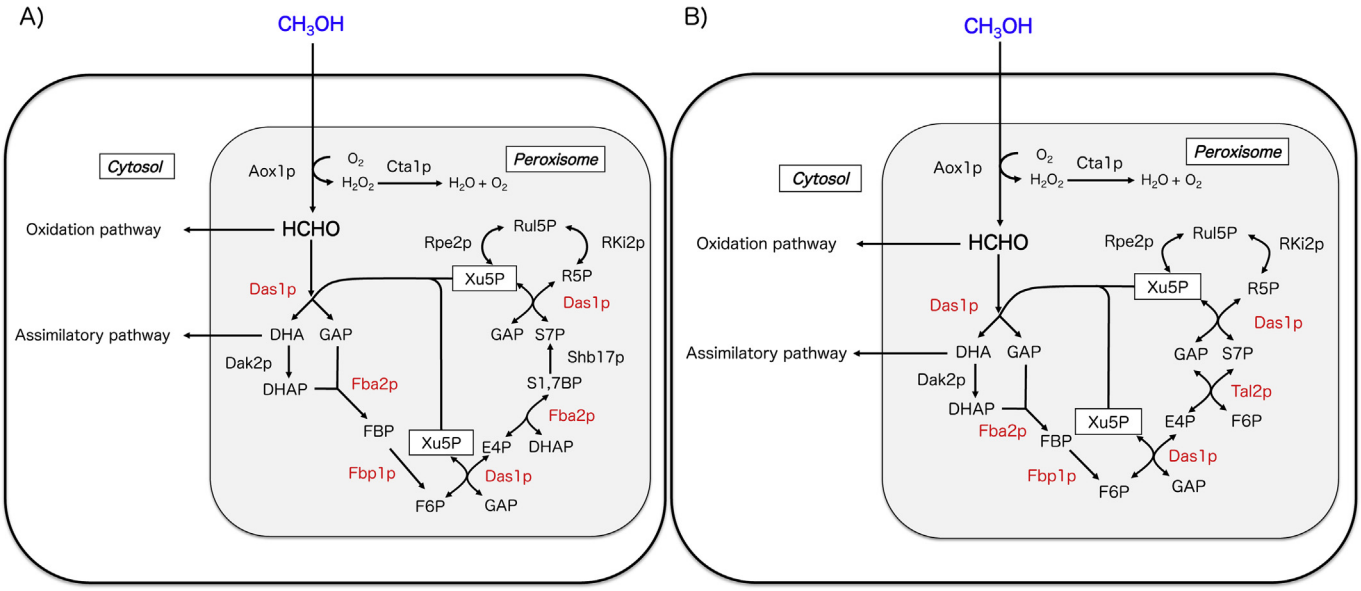

FIG. 1. Schematic representation of the rearrangement pathway for Xu5P in methanol metabolism of *P. pastoris*. (A) Via altered Calvin cycle and (B) via non-oxidative pentose phosphate pathway.

In this study, we aimed to analyze function of individual enzymes/genes, i.e., FBA, FBP, TKL and TAL, in methanol assimilation by gene disruption and promoter expression analyses in *P. pastoris*, in order to validate the evidence of the rearrangement pathway for Xu5P supposed by Ruβmayer et al. (7).

MATERIALS AND METHODS

Strains, media, and cultivation conditions *P. pastoris* strain GS115 (*his4*) was used as the wild-type strain.

Complex YPD medium (2% glucose, 1% Bacto yeast extract, 2% Bacto peptone) and YNB medium (0.67% yeast nitrogen base without amino acids) were used for cultivation of *P. pastoris* strains. The medium was supplemented with following carbon sources: 2% glucose (w/v), 2% glycerol (v/v), 1% methanol (v/v) and 0.5% oleate (v/v). Tween 80 was added to the oleate medium at a concentration of 0.05% (v/v). Histidine (200 μg/ml) and Zeocin (50 μg/ml; Invitrogen, Carlsbad, CA, USA) were supplemented to the media.

Cultivation was done under aerobic conditions at 28°C with rotary shaking, and the growth of the yeast was followed by measuring the optical density at 610 nm.

One-step disruption of the genes Oligo primers used in this study are listed in Table 1.

The Zeocin resistance (*Zeo*^{r}) gene was amplified by PCR with primers, Zeocin-5'-Not-C_7 and Zeocin-3'-$G_5C_5G_5$, yielding the PCR fragment, Not-C_7-*Zeo*^{r}-$G_5C_5G_5$.

For construction of *FBP1* (PAS_chr2-1_0110) gene disruption cassette, a 0.5-kb upstream region of *FBP1* tagged with G_7-*Not* I sequence was amplified by PCR with primers, PpFBP1p-500 and PpFBP1-GGNot, with genomic DNA as the template. A 0.4-kb downstream region of *FBP1* tagged with $C_5G_5C_5$ sequence was amplified with primers PpFBP1+500 and PpFBP1-$C_5G_5C_5$. For construction of *FBA2* (PAS_chr1-1_0319) gene disruption cassette, a 0.5-kb upstream region of *FBA2* tagged with G_7-*Not* I sequence was amplified by PCR with primers, PpFBA2-500 and PpFBA2-G_7Not with genomic DNA as the template. A 0.5-kb downstream region of *FBA2* tagged with $C_5G_5C_5$ sequence was amplified with primers PpFBA2+500 and PpFBA2-$C_5G_5C_5$. For construction of *TAL2* (PAS_chr2-2_0338) gene disruption cassette, a 0.5-kb upstream region of *TAL2* tagged with G_7-*Not* I sequence was amplified by PCR with primers, PpTAL2-500 and PpTAL2-G_7Not with genomic DNA as the template. A 0.5-kb downstream region of *TAL2* tagged with $C_5G_5C_5$ sequence was amplified with primers PpTAL2+500 and PpTAL2-$C_5G_5C_5$. These PCR products and Not-C_7-*Zeo*^{r}-$G_5C_5G_5$ were used for the fusion PCR mediated by GC-rich overlap sequences (10), yielding the *FBP1*, *FBA2*, and *TAL2* disruption cassettes. For construction of *TKL1* (PAS_chr1-4_0150) and *TAL1* (PAS_chr2-2_0337) gene-disruption cassettes, these genes were PCR-amplified from *P. pastoris* genome with PpTKL1infusionF and PpTKl1infusionR, or PpTAL1infusionF and PpTAl1infusionR primer pairs, and cloned into pIB1 (11) by In-Fusion HD cloning kit (Takara Bio Inc., Shiga, Japan). Using the resultant plasmids as templates, inverse PCR was performed to erase the ORF parts, with PpTKL1invF and PpTKL1invR or PpTAL1invF and PpTAL1invR primer pairs. The amplified PCR products were ligated with a gene fragment containing Zeocin-resistance cassette obtained from pGAPZ-A (Invitrogen Japan K.K.) using the In-Fusion HD cloning kit.

These disruption cassettes were transformed into the wild-type strain by electroporation. Zeocin-resistant colonies were selected on SD medium supplemented with histidine and Zeocin or YPD medium containing 100 mg/L Zeocin. Disruption of the *FBP1*, *FBA2*, *TKL1*, *TAL1*, and *TAL2* genes was confirmed by PCR method.

Construction of the strains for promoter assay P_{FBA2}, promoter of *FBA2*, was amplified by PCR with primers, the Bgl-5-PpFBA2p and Sfu-3-PpFBA2p with genomic DNA as the template. The 1.0-kb amplified fragments were subcloned to pT7Blue (Novagen, Madison, WI, USA), yielding pFBA2P. pFBA2P was digested with *Bgl* II and *Sfu* I, and the resulting 1.0-kb fragments was ligated into the *Bgl* II-*Sfu* I site of pPIC6/*LacZ* (Invitrogen Japan K.K.), yielding pPFBA2-LacZ-Bla. pPFBA2-LacZ-Bla was digested with *Bgl* II and *Not* I, and the resulting 4.0-bp fragment was ligated into the *Bgl* II-*Not* I site of pPICZ (Invitrogen Japan K.K.), yielding pP_{FBA2}-LacZ-Zeo.

P_{FBP1}, P_{FBA1}, P_{TKL1}, P_{TKA1}, and P_{TKA2} were amplified by PCR with each primers listed in Table 1 with genomic DNA as the template. The 1.0-kb amplified fragments were subcloned to pT7Blue (Novagen), and yielding plasmids were digested with *Bgl* II and *Nar* I, and with *Bgl* II and *Cla* I, respectively. These promoter fragments were ligated into the *Bgl* II-*Sfu* I site of pP_{FBA2}-LacZ-Zeo, yielding the plasmids for promoter assay.

The linearized plasmids, which were digested with *Sph* I, *Nhe* I, *Hin*d III, *Psh*A I, *Mun* I or *Pst* I within P_{FBP1}, P_{FBA1}, P_{FBA2}, P_{TKL1}, P_{TAL1} or P_{TAL2} promoter region, respectively, were transformed into the wild-type strain by electroporation. Zeocin-resistant colonies were selected on SD medium supplemented with histidine and Zeocin. Yielding strains were named as strains P_{FBP1}-*Z*, P_{FBA1}-*Z*, P_{FBA2}-*Z*, P_{TKL1}-*Z*, P_{TAL1}-*Z* and P_{TAL2}-*Z*. The specific integration of the linearized plasmids was confirmed by PCR method.

Construction of the EGFP-Fba2p and EGFP-Tal2p strains Plasmid pMO201 for expression of mCherry-PTS1 under P_{AOX1} regulation was generated by cloning the PCR-amplified gene fragment encoding mCherry-PTS1 into a backbone vector pIB4-ARG, where *HIS4* of pIB4 (11) had been replaced by *ARG4*. This plasmid was cut with *Aat* II and introduced to a parental strain PPY12, yielding strain MOP101. The plasmids harboring *FBA2* or *TAL2* were generated by cloning the PCR-amplified fragment of *FBA2* (with primers PpFBA2-500infusion and PpFBA2+120infusion) or *TAL2* (with primers PpTAL2-260infusion and PpTAL2+110infusion) into pIB1 (11) cut with *Eco*R I and *Pst* I, using In-fusion HD cloning kit (Takara Bio Inc.). The generated plasmids were used as templates for subsequent inverse PCR: primers PpFBA2invR-EGFPN and PpFBA2invF-EGFPC were used for the reaction toward the *FBA2*-cloned plasmid, and for the *TAL2*-cloned plasmid we used primers PpTAL2invR-EGFPN and PpTAL2invF-EGFPC. The amplified PCR fragments were ligated with a gene fragment encoding EGFP using In-fusion HD cloning kit, yielding pMO202 (possessing P_{FBA2}-*EGFP-PpFBA2ORF*-T_{FBA2}) and pMO203 (possessing P_{TAL2}-*EGFP-PpTAL2ORF*-T_{FBA2}). These plasmids were introduced to the above-mentioned strain MOP101, giving MOP102 (expressing mCherry-PTS1 and EGFP-*Pp*Fba2p) or MOP103 (mCherry-PTS1 and EGFP-*Pp*Tal2p).

Preparation of cell-free extracts and enzyme assays *P. pastoris* strains were grown on YPD medium, and then transferred to the YNB medium containing the described carbon sources. Cell-free extracts from the yeast cells were prepared by the method described previously (12).

TABLE 1. Oligonucleotide primers used in this study.

Primer	Sequence (5′ − 3′)
Zeocin-5'-Not-C7	GCGGCCGCCCCCCCCACACACCATAGCTTC
Zeocin-3'-G5C5G5	GGGGGCCCCCGGGGGTTTGCTCACATG TTGGTCTCCA
PpFBP1-500	AGATCTAAGAATA TGGACAGAGGGAAACAG
PpFBP1-G7Not	GGGGGGGGCGGCCGCGCTAATGTAAGA TAACGTAAGAAGAGAGG
PpFBP1+500	GAATTCACGGGAAGTGGTGGTAAGGG
PpFBP1-G5C5G5	CCCCCGGGGGCCCCCTGCGTTCGA TTGGCACTG
PpFBA2-500	GGCATTGTGACTTGACTTTTACC
PpFBA2-G7Not	GGGGGGGGCGGCCGCAAATTAATCAA TTACCTTATCAAGGTAG
PpFBA2+500	TCCTCAGGATGAAGACGACG
PpFBA2-G5C5G5	CCCCCGGGGGCCCCCGTCTATAGGCTATTA TACTTATCCAGGTAG
PpTKL1infusionF	GACGGCCAGTGAATTCCGAGGGTGGC TCAACAACTA
PpTKL1infusionR	CATGTCTAAGAAGCTTCTACGAGAA TGCAGCTGATGAACATAAGG
PpTAL1infusionF	GACGGCCAGTGAATTCAGAGATCGTG TTTTGATTAAGATTGCTGCT
PpTAL1infusionR	CATGTCTAAGAAGCTTTTACTCCCTTG TTTCCTCGGTGCC
PpTKL1invF	AAGCTATGGTGTGTGTAGAGTGGATG TAGAATACAAGTCTAGAGTTAG
PpTKL1invR	TTTAATTTGCAAGCTAGTAAGTTCATAG TGCGGGAAGAATAGGC
PpTAL1invF	AAGCTATGGTGTGTGGAG TAAAAGAAAGAGCGCAACCCAGGTG
PpTAL1invR	TTTAATTTGCAAGCTGATCCGTGTC TAGCAATGATTGACGTAC
Bgl-5-PpFBP1p	AGATCTTTTGATGATGATTTGTTGTGAACG
Nar-3-PpFBP1p	GGCGCCGCTAATGTAAGATAACG TAAGAAG
Bgl-5-PpFBA1p	AGATCTCTCTACCCAGGATTATTTTTCTTC
Cla-3-PpFBA1p	ATCGATTGTGTAAAAAATATGTTCAATTGA
Bgl-5-PpFBA2p	AGATCTACTCTACCCAGGATTATTTTTCTT
Sfu-3-PpFBA2p	TTCGAAATTTATGAAATTAATCAATTACCT
PpFBA2-500infusion	GACGGCCAGTGAATTCGCCTTTTCCA TTTCGGTTCGGA
PpFBA2+120infusion	CATGTCTAAGAAGCTCAGTAACTACCTGGA TAAGTATAATAGCCT
PpFBA2invR-EGFPN	ACCATGGTGGTCTAGATTTATGAAATTAA TCAATTACCTTATCAAGG
PpFBA2invF-EGFPC	GCTGTACAAGGAATTCTCTACATTTGA TTTCCTTTCCAGAAAAAGC
PpTAL2-260infusionF	GACGGCCAGTGAATTCTGCAACAAAGTA TAAACGGTTGTGAGC
PpTAL2+110infusionR	CATGTCTAAGAAGCTTGCCCTATCAGG TTTGGTTAGC
PpTAL2invR-EGFPN	ACCATGGTGGTCTAGGATATTTCAGCTGAA TTTGAAGATACTCATAC
PpTAL2invF-EGFPC	GCTGTACAAGGAATTCGAATCCAATCCTA TCAACTCTTTATC

FBA activity toward the cleavage of F1,6BP was determined by an NADH-linked enzymatic assay (13). α-Glycerophosphate dehydrogenase-triosephosphate isomerase from rabbit muscle (Sigma−Aldrich Japan K.K., Tokyo, Japan) was used for the assay. One unit of FBA activity was defined as the amount of enzyme catalyzing the cleavage of 1 μmol of F1,6BP and oxidation of 2 μmol of NADH per min.

β-Galactosidase activity was measured by the protocol of Rose and Botstein (14). Units are expressed as nmol of *o*-nitrophenyl β-D-galactoside (ONPG) hydrolyzed per min/mg of protein. One unit of enzyme was defined as the amount of enzyme which liberated 1 nmol of ONPG per min.

The protein concentrations of cell-free extracts were determined by the method of Bradford (15) with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard.

RESULTS

FBP1* is an essential gene for methanol growth of *P. pastoris In our previous work, gene-tagging mutagenesis of *P. pastoris* had been established, leading to generations of *atg* (previously called *paz*) mutant strains deficient in degradation of peroxisomes (16). We employed this technique to obtain methanol-growth deficient mutants, and identified *FBP1* (PAS_chr3_0868) gene, together with *AOX1*, *DAS1*, *CTA1*, *FLD1*, *FGH1*, *FDH1* and *DAK1*, as essential genes for growth on methanol (unpublished data).

In order to confirm the function of *FBP1* gene on methanol metabolism, we constructed the gene-disrupted strain of *FBP1*. Strain *fbp1*Δ could not grow on methanol as a sole carbon source, although the strain showed normal growth on glucose (Fig. 2A, B). The strain also exhibited significant growth defect on glycerol or oleate, as with the case of methanol medium (Fig. 2C, D). Moreover, gene expression of *FBP1* was upregulated by non-fermentative carbon sources, glycerol, oleate and methanol (Fig. 3). Especially methanol was the best inducer for the *FBP1* expression, and its expression level by methanol was about tenfold higher than that by glucose (Fig. 3).

These results indicate that *FBP1* has an indispensable physiological role on methanol metabolism of the methylotrophic yeast, together with genes for other methanol-metabolic enzymes.

***FBA2* encodes methanol-inducible FBA, although *FBA1* is constitutive gluconeogenesis gene** Because FBA catalyzes the synthesis of F1,6BP, which is a substrate for FBP, it seems that FBA is an essential enzyme for methanol metabolism. Indeed, FBA activity in *P. pastoris* was induced strongly by methanol, compared with glucose, oleate and glycerol (Fig. 4A). Thus FBA is also one of the methanol-inducible enzymes, such as Fbp1p, Aox1p and Das1p. In the genome of *P. pastoris*, however, there are two copies of the FBA genes, *FBA1* (PAS_chr1-1_0072) and *FBA2* (PAS_chr1-1_0319), which showed high homology to each other (74% identity) and to FBAs from other yeast strains. In order to observe expression levels of *FBA1* and *FBA2* separately, we constructed strains P_{FBA1}-*Z* and P_{FBA2}-*Z*, which possessed P_{FBA1}-and P_{FBA2}-*LacZ* expression cassettes, respectively. As a result of promoter assay, it was revealed that *FBA1* was constitutively expressed on all cells grown on several carbon sources (Fig. 4B). This result indicates that *FBA1* encodes a standard type of FBA, which participates in gluconeogenesis, and we concluded that *FBA1* might be a lethal gene for the methylotrophic yeast, like FBA in *Saccharomyces cerevisiae* (17,18). On the other hand, expression of *FBA2* was observed only in methanol-grown cells (Fig. 4B). Therefore, *FBA2* encodes a specific FBA for methanol metabolism. Indeed, the growth rate of strain *fba2*Δ on methanol was slightly lowered, although the strain could grow on methanol (Fig. 5).

***TKL1* does not participate in methanol metabolism** In the second stage of the rearrangement pathway for Xu5P, it seems that TKL, which acts in the non-oxidative pentose phosphate pathway, is one of the essential enzymes for methanol metabolism of the yeast, because TKL-catalyzed reaction synthesizes directly Xu5P from F1,6BP and D-glyceraldehyde 3-phosphate (GAP). In the genome of *P. pastoris*, there is only one copy of the *TKL* gene, *TKL1* (PAS_chr1-4_0150), except for *DAS* genes.

In order to observe the expression level of *TKL1*, we constructed strains P_{TKL1}-*Z*, which possessed a P_{TKL1}-*LacZ* expression cassette. As a result of promoter assay, it was revealed that expression of *TKL1* was slightly repressed by methanol (Fig. 6A). Moreover, strain *tkl1*Δ on methanol could grow on methanol as well as the wild type strain (Fig. 5). From these facts, Tkl1p does not participate in methanol metabolism of the yeast.

***TAL2* encodes methanol-inducible TAL, although *TAL1* is constitutively expressed** TAL catalyzes the next reaction step of TKL in the non-oxidative pentose phosphate pathway, and it

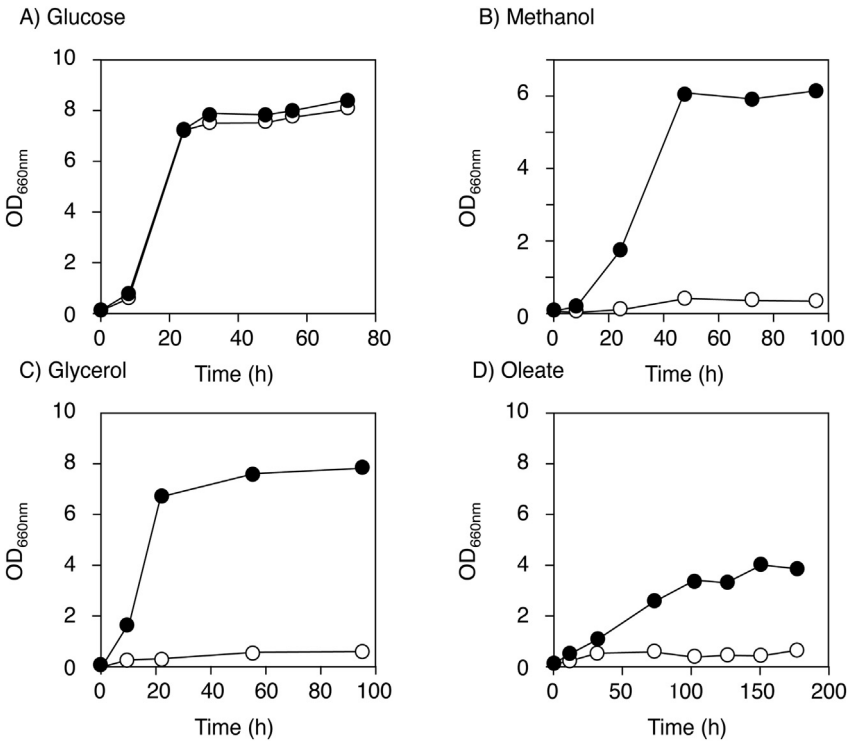

FIG. 2. Growth of strains wild-type (closed circles) and *fbp1*Δ (open circles) on (A) glucose, (B) methanol, (C) glycerol, or (D) oleate as a sole carbon source.

converts D-sedoheptulose 7-phosphate (S7P) and GAP to D-erythrose 4-phosphate (E4P) and F6P. There are two copies of the *TAL* genes, *TAL1* (PAS_chr2-2_0337) and *TAL2* (PAS_chr2-2_0338), which showed high homology with each other (73% identity at amino acid level) and with *TAL*s from other yeast strains, in the genome of *P. pastoris*.

In order to observe expression levels of *TAL1* and *TAL2* separately, we constructed strains P_{TAL1}-*Z* and P_{TAL2}-*Z*, which possessed P_{TAL1}-and P_{TAL2}-*LacZ* expression cassettes, respectively. As a result of promoter assay, it was revealed that *TAL1* was constitutively expressed on all cells grown on several carbon sources (Fig. 6B). It is suggested that *TAL1* encodes a standard type of TAL. On the other hand, expression of *TAL2* was observed on only methanol-grown cells (Fig. 6B). From these findings, *TAL2* may encode a specific TAL for methanol metabolism. However, strain *tal2*Δ could grow on methanol well, and did not show any growth defect as with the case of strain *tal1*Δ (Fig. 5).

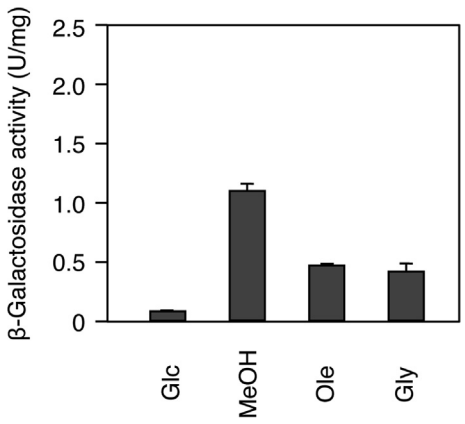

FIG. 3. Induction pattern of β-galactosidase activity in the P_{FBP1}-*Z* strain. Glc, glucose; MeOH, methanol; Ole, oleate; Gly, glycerol.

Fba2p and Tal2p are peroxisomal enzymes It was reported that, in general, FBA, FBP, TKL and TAL is localized in cytosol in several yeast strains (19–21). Fba2p and Tal2p, however, possess a C-terminal tripeptide SKL, which acts as the peroxisome targeting signal type 1 (PTS1) in *P. pastoris*, although Fba1p, Tkl1p and Tal1p do not possess any PTSs. In methanol grown cells, EGFP-Fba2p and EGFP-Tal2p were colocalized with mCherry-SKL in the peroxisomes (Fig. 7). From these findings, it was suggested that Fba2p and Tal2p are peroxisomal proteins, together with Aox1p, Das1p and Cta1p.

DISCUSSION

The C_1-acceptor molecule, such as Xu5P, is an indispensable compound in the methanol metabolism of the methylotrophic yeast, and the rearrangement pathway for Xu5P is also essential pathway for yeast methylotrophy. Recently, Ruβmayer et al. (7) proposed metabolic map for the rearrangement pathway for Xu5P in methanol metabolism (Fig. 1A). In this study, in order to validate the rearrangement pathway suggested by Ruβmayer et al. (7), we analyzed function of individual enzymes/genes, i.e., FBAs, FBPs, TKL and TALs, in the rearrangement pathway by gene disruption and promoter expression analyses.

As a result, our data confirmed the rearrangement pathway for Xu5P proposed by Ruβmayer et al. (7), because (i) *FBP1*, *FBA2* and *TAL2* were methanol inducible genes, together with *AOX1*, *DAS1* and other genes encoding methanol-metabolic enzymes, (ii) Fba2p and Tal2p were localized in peroxisomes, (iii) strain *fbp1*Δ showed severe growth defect on methanol and non-fermentable carbon

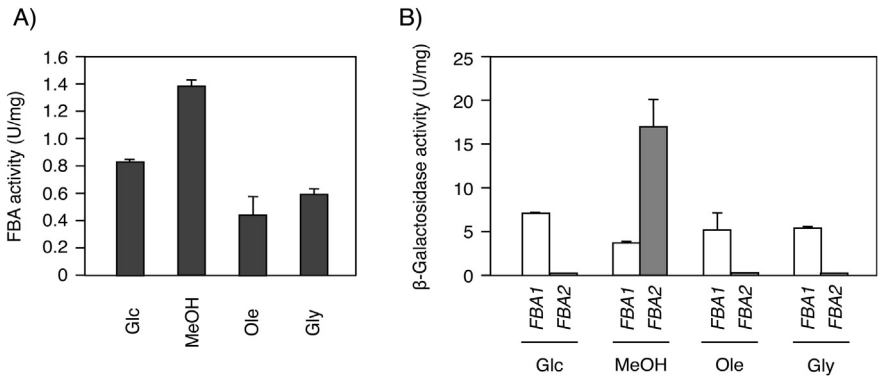

FIG. 4. Induction pattern of (A) FBA activity in the wild type strain, and (B) β-galactosidase activities in the P_{FBA1}-*Z* and P_{FBA2}-*Z* strains. Glc, glucose; MeOH, methanol; Ole, oleate; Gly, glycerol.

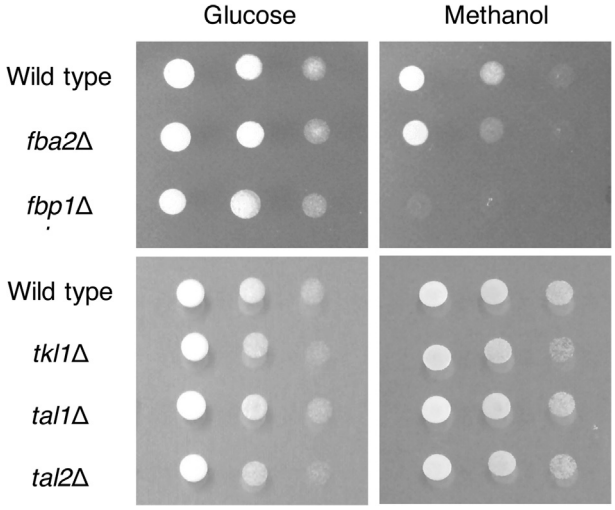

FIG. 5. Growth of *P. pastoris* gene disrupted strains on the glucose or methanol plates. Strains wild-type, *fbp1*Δ and *fba2*Δ, *tkl1*Δ, *tal1*Δ and *tal2*Δ.

sources, and (iv) the growth rate of strain *fba2*Δ in methanol medium was slightly decreased. On the other hand, *TKL1* was not methanol-inducible gene (Fig. 6A), although TKL reaction is one of the indispensable steps in the rearrangement pathway for Xu5P. Moreover, Tkl1p does not have PTS1 and its localization is not in peroxisomes (7), despite all other members are peroxisomal proteins. This contradiction can be explained by enzymatic properties of DAS localized in peroxisomes. It was reported that DAS has the classical TKL activity (8,9), thus DAS can catalyze the synthesis of Xu5P in peroxisomes, instead of classical TKL. That is to say, the entire rearrangement pathway for Xu5P locates in the peroxisomes, together with AOD and DAS, which are enzymes to oxidize methanol and to fix carbon molecule from methanol to Xu5P. These findings imply that all steps for methanol assimilation completes within the peroxisome matrix. The localization of the methanol assimilation pathway may be certainly reasonable, because in this system, it is not necessary to transport sugar phosphate into peroxisomes from cytosol across the membrane.

Ruβmayer et al. (7) proposed that the metabolic rearrangement for the supply of Xu5P proceeds through the altered Calvin cycle, utilizing S1,7BP as the intermediate (Fig. 1A). In this study we found that strain *fba2*Δ was still able to grow on methanol (Fig. 5), which is difficult to be rationalized by simply hypothesizing the altered Calvin cycle, since this pathway requires Fba2p in several steps therein, making this enzyme a pivotal factor (Fig. 1A). Rather, growth phenotypes of the mutants (Fig. 5) strongly suggested that the altered Calvin cycle and non-oxidative pentose phosphate pathway (involving Tal2p) (Fig. 1B) function in a complementary manner.

As described above, it is concluded the rearrangement pathway for Xu5P contains the duplicate methanol inducible enzyme sets and the pathway is entirely localized within peroxisomes. These facts teach us a renewed recognition that peroxisomes have indispensable functions for methanol metabolism of the methylotrophic yeast.

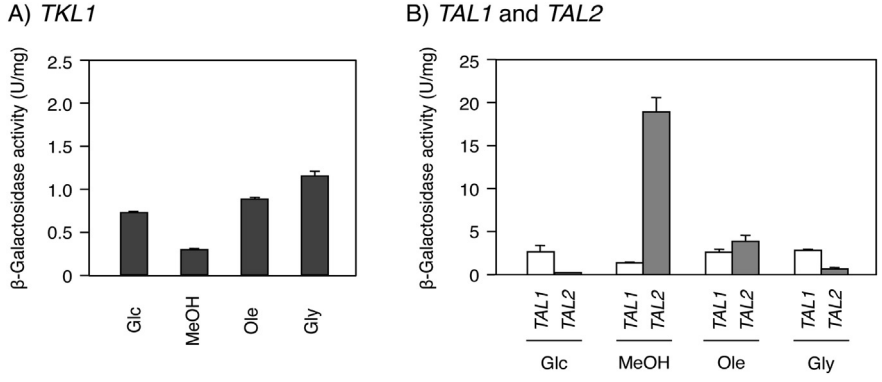

FIG. 6. Induction pattern of β-galactosidase activity under the control of (A) P_{TKL1}, and (B) P_{TAL1} and P_{TAL2}. Glc, glucose; MeOH, methanol; Ole, oleate; Gly, glycerol.

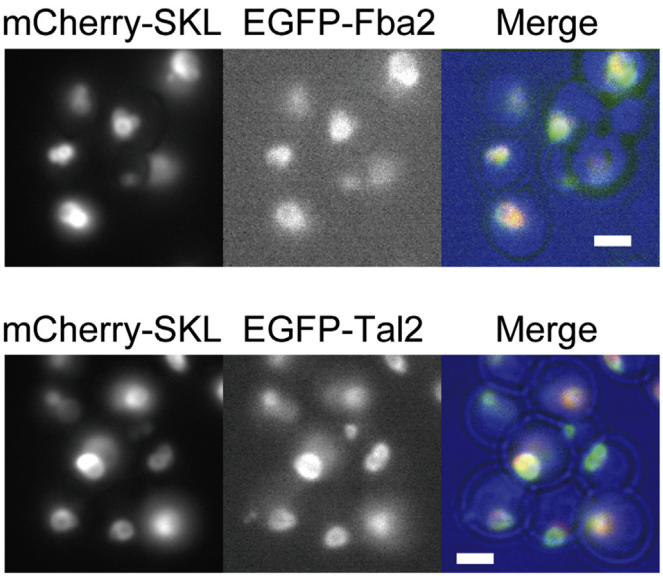

FIG. 7. Subcellular localization of EGFP-Fba2p and EGFP-Tal2p. Strains MMOP202 and MOP203 were cultured in synthetic methanol medium at 28°C for 20 h and subjected to fluorescence microscopy. The Merge images consist of red mCherry-PTS1, green EGFP, and blue brightfield images. Bar: 2 μm.

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