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Regulation and Physiological Role of the *DAS1* Gene, Encoding Dihydroxyacetone Synthase, in the Methylotrophic Yeast *Candida boidinii*

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The physiological role of dihydroxyacetone synthase (DHAS) in *Candida boidinii* was evaluated at the molecular level. The *DAS1* gene, encoding DHAS, was cloned from the host genome, and regulation of its expression by various carbon and nitrogen sources was analyzed. Western and Northern analyses revealed that *DAS1* expression was regulated mainly at the mRNA level. The regulatory pattern of DHAS was similar to that of alcohol oxidase but distinct from that of two other enzymes in the formaldehyde dissimilation pathway, glutathione-dependent formaldehyde dehydrogenase and formate dehydrogenase. The *DAS1* gene was disrupted in one step in the host genome (*das1*Δ strain), and the growth of the *das1*Δ strain in various carbon and nitrogen sources was compared with that of the wild-type strain. The *das1*Δ strain had completely lost the ability to grow on methanol, while the strain with a disruption of the formate dehydrogenase gene could survive (Y. Sakai et al., J. Bacteriol. 179:4480–4485, 1997). These and other experiments (e.g., those to determine the expression of the gene and the growth ability of the *das1*Δ strain on media containing methylamine or choline as a nitrogen source) suggested that *DAS1* is involved in assimilation rather than dissimilation or detoxification of formaldehyde in the cells.

In methylotrophic yeasts, formaldehyde is the key intermediate in methanol metabolism since it stands at the branchpoint of pathways for methanol assimilation and dissimilation. Since formaldehyde is an extremely toxic compound, its intracellular level should be strictly regulated.

Dihydroxyacetone synthase (DHAS) (EC 2.2.1.3) catalyzes the first reaction in the assimilation pathway by fixing formaldehyde to D-xylulose 5-phosphate, after formaldehyde is generated from methanol via alcohol oxidase (AOD) (EC 1.1.3.13) (3, 11). Otherwise, in a formaldehyde oxidation pathway, formaldehyde is dissimilated to CO_2 through enzymes, e.g., glutathione-dependent formaldehyde dehydrogenase (FLD) (EC 1.2.1.1) and formate dehydrogenase (FDH) (EC 1.2.1.2).

So far, the physiological significance of these methanol-metabolizing enzymes has been estimated mainly from the enzymatic properties of purified enzyme or from phenotypes of mutants deficient in the specific enzyme obtained via random mutagenesis. Through such analyses, for example, FDH had been considered to be essential to the energy supply for methylotrophic growth (2, 34). However, through gene disruption analysis with *Candida boidinii* (23), the physiological role of FDH was revealed to be mainly detoxification of formate rather than stimulated energy generation. Such unexpected results with FDH led us to reevaluate the physiological roles of a key enzyme in formaldehyde assimilation, DHAS, using a gene-disrupted strain.

Similar to other cases, the physiological function of DHAS as a methanol assimilation enzyme has been estimated from its enzymatic properties and by analysis of a mutant obtained by random mutagenesis (13, 14). However, our previous study showed that loss of a peroxisome membrane protein, Pmp47, not only inhibits transport of DHAS into peroxisomes but also leads to loss of DHAS activity (26), raising the possibility that a previously derived DHAS-deficient mutant strain (13, 14) did not represent the specific mutation in the DHAS structural gene. In addition, we needed to clone and disrupt the gene for DHAS in *C. boidinii* to further investigate the molecular mechanism of peroxisomal transport of DHAS in relation to the function of Pmp47. Furthermore, although the strong and methanol-inducible promoter of the DHAS-encoding gene is expected to be applicable to expression of various heterologous genes in methylotrophic yeasts (37), the regulation of DHAS has not been studied in detail.

This study was conducted (i) to see whether DHAS is involved in detoxification of formaldehyde and (ii) to reveal how DHAS synthesis is regulated by various carbon and nitrogen sources. First, *C. boidinii DAS1*, encoding DHAS, was cloned from the *C. boidinii* genome and its primary structure was determined. Next, the regulation of *DAS1* expression by various carbon and nitrogen sources was investigated and compared with that of other methanol-metabolizing enzymes. Lastly, the *das1*Δ strain, a mutant of *C. boidinii* harboring disrupted *DAS1*, was constructed and used to study the physiological importance of the enzyme in growth on various carbon and nitrogen sources. Our results suggest that DHAS is involved mainly in the assimilation of formaldehyde and that the physiological significance of DHAS for formaldehyde detoxification is minor.

MATERIALS AND METHODS

Yeast and bacterial strains, media, and cultivation. *C. boidinii* TK62 (*ura3*) (22), which was derived from *C. boidinii* S2, was used as the host for mutagenesis. *C. boidinii* GC, which is a *URA3* gene convertant from strain TK62 (25), was used as the wild-type control strain. *Escherichia coli* JM109 (29) was used for plasmid preparation and for the construction of a *C. boidinii* genomic library.

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Complex YPD and synthetic MI media were used for cultivation of *C. boidinii* strains (24). YPD medium consisted of 1% (wt/vol) Bacto-Yeast Extract and 2% (wt/vol) Bacto-Peptone (Difco Laboratories, Detroit, Mich.) and 2% glucose. Synthetic MI medium consisted of 0.28% (wt/vol) KH_2PO_4, 0.06% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.045% (wt/vol) EDTA · 2Na, 0.0055% (wt/vol) $CaCl_2 \cdot 2H_2O$, 0.004% (wt/vol) $FeCl_2 \cdot 6H_2O$, 0.00085% (wt/vol) $MnSO_4 \cdot 3H_2O$, 0.0011% (wt/vol) $ZnSO_4 \cdot 7H_2O$, 0.0002% (wt/vol) $CuSO_4 \cdot 5H_2O$, 0.00014% (wt/vol) $CoCl_2 \cdot 2H_2O$, 0.00013% (wt/vol) $Na_2MoO_4 \cdot 2H_2O$, 0.0002% (wt/vol) H_3PO_3, 0.00003% (wt/vol) KI, and carbon and nitrogen sources. The carbon source was one of the following, unless stated otherwise: 2% (wt/vol) glucose, 2% (vol/vol) glycerol, 1% (vol/vol) methanol, 0.5% (vol/vol) oleic acid, or 0.6% (wt/vol) D-alanine. Tween 80 (Sigma Chemical, St. Louis, Mo.) was added to oleic acid medium at a concentration of 0.05% (vol/vol). The nitrogen source used was one of the following: 0.76% (wt/vol) ammonium chloride, 0.5% (wt/vol) methylamine hydrochloride, or 0.3% (wt/vol) choline chloride. The initial pH of the medium was adjusted to 6.0. Cultivation was performed aerobically at 28°C with reciprocal shaking, and growth was monitored by measuring the optical density at 610 nm. Methanol induction of the *das1*Δ strain was performed by transferring YPD-grown cells to methanol-MI medium at an optical density at 610 nm of 0.5 and subsequently incubating them for 16 h.

Preparation of crude extracts and enzyme assays. Yeast cells were broken on a 3110BX mini-beadbeater (Biospec Products, Bartlesville, Okla.) in buffer A containing glass beads (diameter, 0.5 mm). Buffer A consisted of 50 mM potassium phosphate (pH 7.0), 5 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 1 mM dithiothreitol, 1 mM EDTA, and 0.024% phenylmethylsulfonylfluoride. Glass beads and cell debris were removed by centrifugation at 12,000 × *g* for 10 min at 4°C. DHAS activity was determined as described previously by using β-hydroxypyruvate as the substrate (38). Formaldehyde was determined by the method of Nash (19). One unit of DHAS activity was defined as the amount of protein required to consume 1 μmol of formaldehyde in 1 min.

AOD (35), FLD (33), FDH (33), and catalase (CTA) (1) activities were determined as described previously, and the enzyme activity was defined according to the literature in each case. Protein was determined by the method of Bradford with a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.) by using bovine serum albumin as the standard.

Protein methods. Standard 10% Laemmli gels (15), with the separating gel at pH 9.2, were employed. Immunoblotting was performed by the method of Towbin et al. (36) with the ECL detection kit (Amersham, Arlington Heights, Ill.). Anti-DHAS polyclonal antibody was as described previously (26).

Determination of the N-terminal amino acid sequence. DHAS was purified by the method of Kato et al. (12). The N-terminal amino acid sequence of the purified enzyme was determined by automated Edman degradation by using a Shimadzu PSQ-2 protein sequencer (Shimadzu, Kyoto, Japan).

DNA and RNA methods. Yeast DNA was purified by the method of Cryer et al. (4) or Davis et al. (5). Total RNAs were extracted from *C. boidinii* cells by using ISOGEN (Nippon Gene, Toyama, Japan). Southern and Northern analyses were performed as described previously (22, 27). The gel-purified DNA fragment was ^{32}P labeled by the random primer method (6). The 1.8-kb *Eco*RV-*Bgl*II fragment harboring the *C. boidinii DAS1* coding region and the 0.9-kb *Cla*I-*Hin*dIII fragment harboring the *C. boidinii ACT1* (actin) coding region (26) were used for Northern analyses.

Cloning and sequencing of the *C. boidinii DAS1* gene. In order to construct a pBluescript II KS+ gene library (Stratagene, La Jolla, Calif.), *C. boidinii* S2 genomic DNA was digested with *Eco*RI. The DNA fragments corresponding to approximately 8.3 kb were inserted into the *Eco*RI site of pBluescript II KS+ and transformed into *E. coli* JM109. Transformants were transferred onto a Biodyne nylon membrane (Pall Bio Support, East Hills, N.Y.). After lysis of the transformants and binding of the liberated DNA to nylon membrane, these blots were colony hybridized by using a ^{32}P-labeled partial DHAS cDNA (the 0.5-kb fragment harboring the 3′ half from the second *Xba*I site in the coding region to the poly(A) tail) as the probe. This DHAS cDNA clone, obtained during screening for methanol-inducible peroxisomal genes, was a generous gift from J. M. Goodman, University of Texas, Southwestern Medical Center at Dallas (8). Positive clones were found to harbor a reactive 8.3-kb *Eco*RI fragment, and the recovered plasmid was named pDAS1. pDAS1 was sequenced by using a PRISM DyeDeoxy Terminator Cycle sequencing kit and a model 373A DNA sequencer (Applied BioSystems, Foster City, Calif.) or by the dideoxy termination method of Sanger et al. (30).

Disruption and expression of the *DAS1* gene. Transformation of *C. boidinii* TK62 and the *das1*Δ *ura3* strain was performed by the modified lithium acetate method (21). pDAS1 DNA was digested with *Eco*RV to liberate the 3.8-kb fragment, including most of the DHAS-encoding region. The remaining linear 7.5-kb fragment and the 4.3-kb *Sac*I-*Xho*I fragment of *C. boidinii URA3* DNA from pSPR (28) were made blunt ended with T4 polymerase (Takara Co. Ltd., Kyoto, Japan) and then ligated, yielding the *DAS1* disruption vector, pDASΔ. pDASΔ had the *C. boidinii URA3* DNA as a selectable marker and the truncated *DAS1*-flanking sequences. After digestion of pDASΔ with *Pst*I and *Sal*I, the liberated 8.8-kb fragment was used to transform *C. boidinii* TK62 to uracil prototrophy. The gene disruption was confirmed by genomic Southern analysis using *Eco*RI-digested genomic DNA from each transformant and the ^{32}P-labeled 1.8-kb *Eco*RI-*Eco*RV fragment harboring the 5′-flanking region of *DAS1* as the probe. The *das1*Δ strain was reverted to uracil auxotrophy after 5-fluoroorotidic acid selection, yielding the *das1*Δ *ura3* strain, by our previously described procedure (28). The *das1*Δ *aod1*Δ strain was derived by replacing a 1,579-bp *Sty*I fragment within the *AOD1* coding region (27) of the *das1*Δ *ura3* strain with the 4.3-kb *Sac*I-*Xho*I fragment of *C. boidinii URA3* DNA from pSPR (28).

The *DAS1* expression plasmid was constructed by introducing the PCR-amplified *DAS1* coding region, having two flanking *Not*I sites, into pNoteI (20). The primers used for PCR amplification were as follows: forward primer, 5′GCGG CCGCAAATGGCTCTCGCAAAAGCTGC3′; reverse primer, 5′GCGGCCGC TTATAAATGATTTTGATCATGTTTTG3′. The identity of the PCR product obtained was confirmed by DNA sequence analysis. The constructed plasmid had the *DAS1* coding gene under control of the *C. boidinii AOD1* promoter and the *C. boidinii URA3* gene. The plasmid was linearized with *Bam*HI and introduced into the *C. boidinii das1*Δ *ura3* strain.

Nucleotide sequence accession number. The nucleotide sequence of *DAS1* has been submitted to GenBank and assigned accession no. AF086822.

RESULTS AND DISCUSSION

Primary structure of *C. boidinii DAS1*. During sequencing of the cDNA library clones obtained from methanol-grown *C. boidinii* ATCC 32195 (8), Goodman et al. had found a partial clone that coded for an open reading frame (ORF) similar to the deduced amino acid sequence of *Hansenula polymorpha* DHAS (8a, 10) (unpublished data). Using this putative *DAS1* fragment as the probe, we obtained the complete *DAS1* clone from the genomic library of *C. boidinii* S2. *DAS1* consists of a 2,118-bp ORF corresponding to a protein of 706 amino acid residues (Fig. 1). This ORF was identified as the gene encoding DHAS based on (i) the identity of the N-terminal amino acid sequence, NH_2-ALAKAASINDDIHDLTMRAFR-, derived with that of the purified DHAS protein; (ii) agreement of the calculated molecular mass of this protein (78,132 Da) with that of the purified DHAS as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (78 kDa); and (iii) the loss of DHAS activity in the *das1*Δ strain (described below). The sequence encoded by the *DAS1* coding region showed greater similarity to the deduced amino acid sequence of the *H. polymorpha DAS* product (69% identity) (10) than to the sequences of other transketolases from *Saccharomyces cerevisiae* (7, 31) and *Pichia stipitis* (17) (39 to 41% identity) (Fig. 1). All of these transketolases contained transketolase signature 1 (amino acid residues 22 to 42) (16), transketolase signature 2 (residues 482 to 517) (16, 32), the catalytic domain of transketolase (residues 418 to 434) (7), and a possible thiamine pyrophosphate binding domain (residues 165 to 196) (9, 32) (Fig. 1).

Regulation of *DAS1* expression by various carbon and nitrogen sources. The activities of methanol-metabolizing enzymes in *C. boidinii* cells grown on different carbon and nitrogen sources were studied. As shown in Table 1, when NH_4Cl was used as the sole nitrogen source for growth, DHAS activity was induced by methanol but was not induced by glucose, glycerol, or other peroxisome-inducing carbon sources, i.e., oleate and D-alanine. DHAS induction by methanol was repressed by glucose (Table 1; glucose + methanol) but not by glycerol (Table 1; glycerol + methanol). Methylamine and choline, when used as nitrogen sources, are known to be metabolized to formaldehyde in yeast cells (18). When either of these substrates was used as a nitrogen source together with glycerol as the carbon source, we observed induction of DHAS activity (Table 1; MA/glycerol or Chl/glycerol). In contrast, when glucose was used as the carbon source, DHAS activity was not induced (Table 1; MA/glucose or Chl/glucose). These results indicate that DHAS activity was induced by methanol or formaldehyde and that induction of DHAS activity suffers from repression by glucose but not by glycerol.

As shown in Table 1, regulation of DHAS was more like that of AOD than the other two enzymes, FDH and FLD, both involved in the formaldehyde oxidation pathway; i.e., AOD

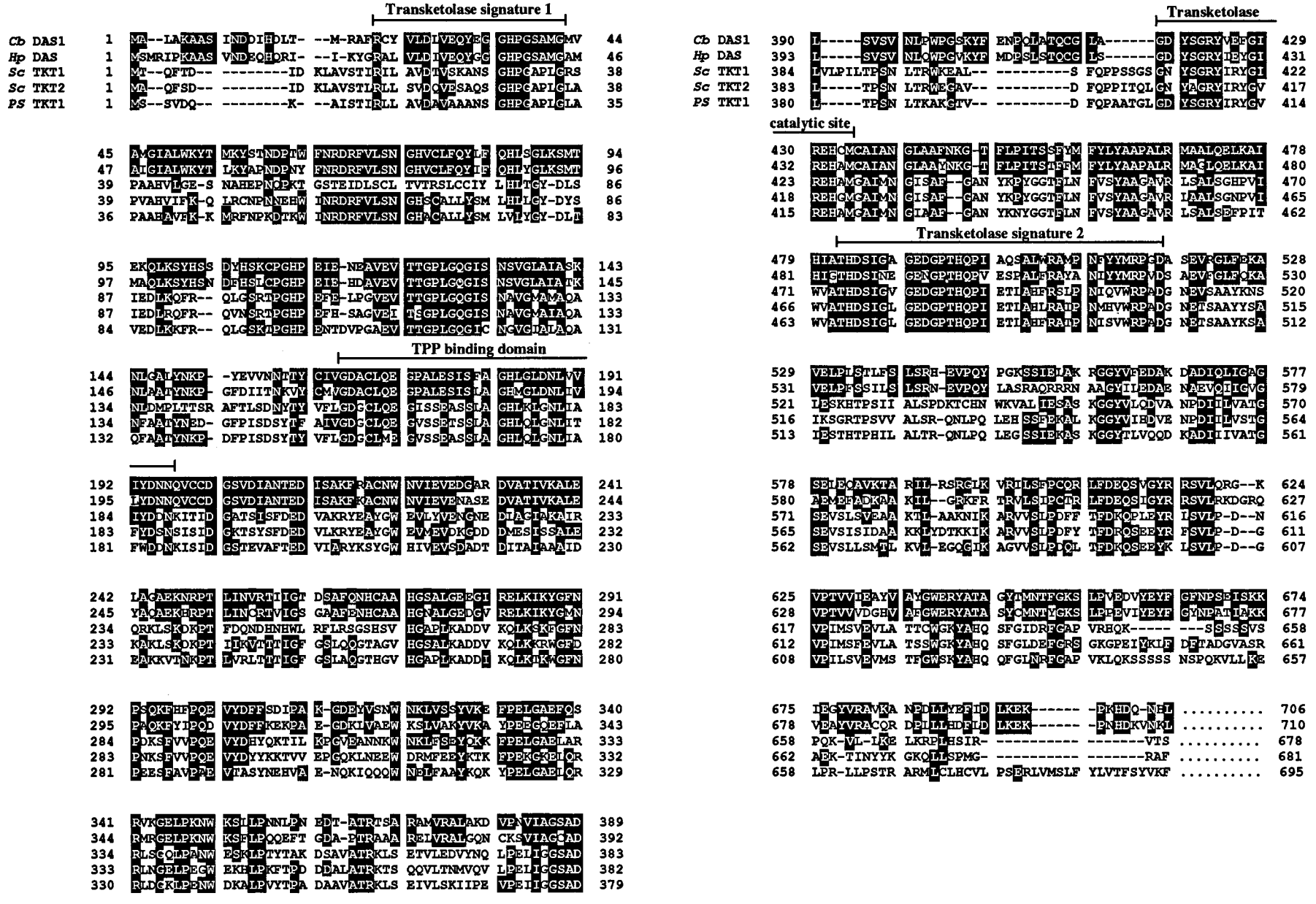

FIG. 1. Alignment of the deduced amino acid sequence of the *C. boidinii DAS1* (*Cb DAS1*) product with sequences of *H. polymorpha* DHAS (*Hp* DAS) (GenBank accession no. X02424 [10]), *S. cerevisiae* transketolase 1 (*Sc* TKT1) (GenBank accession no. X73224 [31]) and transketolase 2 (*Sc* TKT2) (GenBank accession no. X73532 [31]), and *P. stipitis* transketolase (*Ps* TKT1) (GenBank accession no. Z26486 [17]). White letters indicate amino acid residues identical to those of the *C. boidinii DAS1* product.

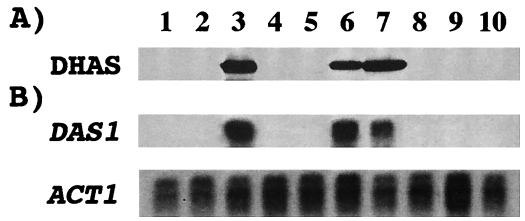

FIG. 2. Regulation of *DAS1* expression in *C. boidinii*. (A) Western analysis. Protein (20 μg) was separated by SDS-PAGE and detected with anti-DHAS polyclonal antibodies. (B) Northern analysis. Total RNA (20 μg) was loaded in each lane and probed with either the ^{32}P-labeled *DAS1* or the ^{32}P-labeled *ACT1* (actin) DNA as described in Materials and Methods. Carbon sources in each lane are as follows: lanes 1, 8, and 9, glucose; lanes 2, 6, and 7, glycerol; lane 3, methanol; lane 4, oleate; lane 5, D-alanine; and lane 10, glucose plus methanol. Nitrogen sources in each lane are as follows: lanes 1, 2, 3, 4, 5, and 10, NH_4Cl; lanes 6 and 8, methylamine; and lanes 7 and 9, choline. The concentrations of carbon and nitrogen sources are described in Materials and Methods.

TABLE 1. Relative activities of enzymes related to methanol metabolism during growth on various carbon and nitrogen sources[a]

N source/C source	Relative activity (%)			
	DHAS	AOD	FDH	FLD
NH_4Cl/methanol	100	100	100	92
NH_4Cl/glucose	ND[b]	ND	ND	ND
NH_4Cl/glycerol	ND	20	3.3	22
NH_4Cl/oleate	ND	ND	4.4	4.5
NH_4Cl/D-alanine	ND	ND	3.3	25
NH_4Cl/glucose + methanol	ND	ND	ND	ND
NH_4Cl/glycerol + methanol	67	90	32	67
MA[c]/glycerol	40	32	36	80
Chl[d]/glycerol	42	63	49	100
MA/glucose	ND	ND	31	89
Chl/glucose	ND	ND	78	75

[a] Cells were grown and disrupted as described in Materials and Methods. Specific activities of DHAS, AOD, FLD, and FDH for methanol medium are 0.22 ± 0.06, 2.3 ± 0.18, 0.82 ± 0.10, and 0.55 ± 0.08 U/mg of protein, respectively.
[b] ND, not detected.
[c] MA, methylamine.
[d] Chl, choline.

and DHAS showed complete repression by glucose, while FLD and FDH did not, when formaldehyde was generated in the cells via methylamine or choline metabolism.

Next, *DAS1* expression was monitored at the protein and mRNA levels. We conducted Western and Northern analyses by using crude extracts and total RNAs extracted from *C. boidinii* cells grown on each carbon and nitrogen source (Fig. 2). These regulatory patterns of *DAS1* expression and the regulatory pattern of DHAS enzyme activity (Table 1) coincided each other. Therefore, the regulation of DHAS activity was confirmed to be controlled mainly at the mRNA level.

Disruption of the *DAS1* gene causes defects in growth on methanol and glycerol-plus-methanol media. Disruption of the *DAS1* gene was confirmed by Southern analysis with *Eco*RI-digested DNA from each transformant (Fig. 3A). The DNA from the wild-type strain gave a signal of 8.3 kb; this signal shifted to 8.8 and 5.5 kb in the *das1*Δ and *das1*Δ *ura3* strains, respectively, as expected for disruption and deletion of the *URA3* sequence, caused by a homologous recombination (Fig. 3B). Methanol-induced cells of the *das1*Δ strain did not show any DHAS activity but exhibited AOD (1.8 U/mg of protein), CTA (2,611 U/mg of protein), FLD (0.62 U/mg of protein), and FDH (0.40 U/mg of protein) activities comparable to the levels for the wild-type strain (Table 1). Western analysis using anti-DHAS antibody showed no signal in the cell extracts of the *das1*Δ strain and the *das1*Δ *ura3* strain (Fig. 3C).

The *das1*Δ strain had lost the ability to grow on methanol

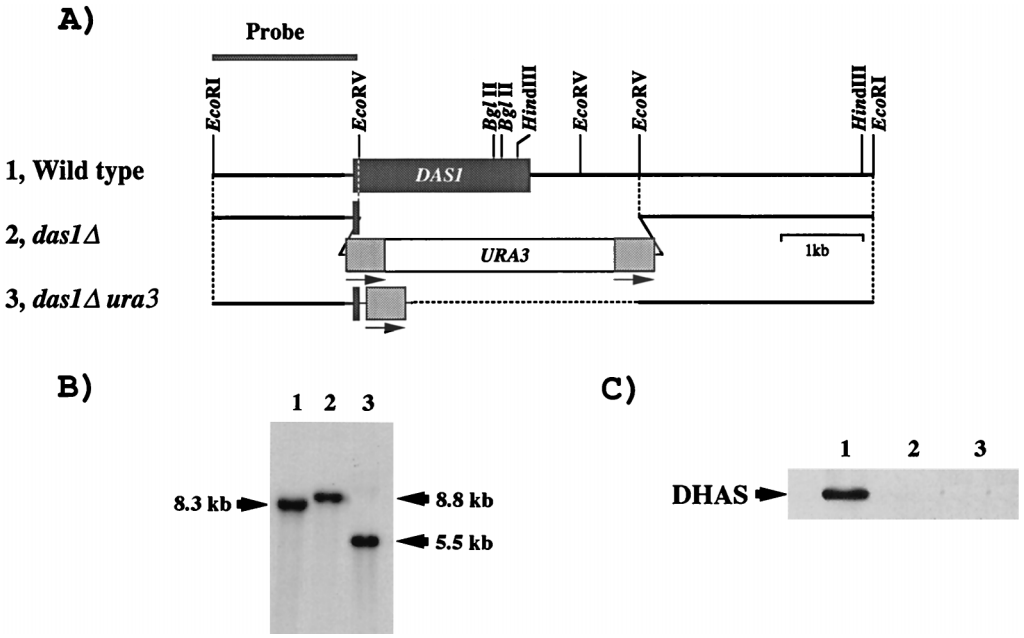

FIG. 3. One-step disruption of the *DAS1* gene in *C. boidinii*. (A) Restriction map of the cloned fragment and disruption strategy. The lightly shaded boxes and arrows at both ends of *URA3* show repeated sequences for homologous recombination to remove the *URA3* gene after the gene disruption. (B) Genomic Southern analysis of *Eco*RI-digested total DNAs (3 μg of each) from the host strain TK62 (lane 1), the *das1*Δ strain (lane 2), and the *das1*Δ *ura3* strain (lane 3) probed with the ^{32}P-labeled 1.8-kb *Eco*RI-*Eco*RV fragment, including the 5′-flanking region of *DAS1*. (C) Immunoblot analysis of strain TK62 (lane 1), the *das1*Δ strain (lane 2), and the *das1*Δ *ura3* strain (lane 3) with extracts of methanol-induced cells and anti-DHAS polyclonal antibody.

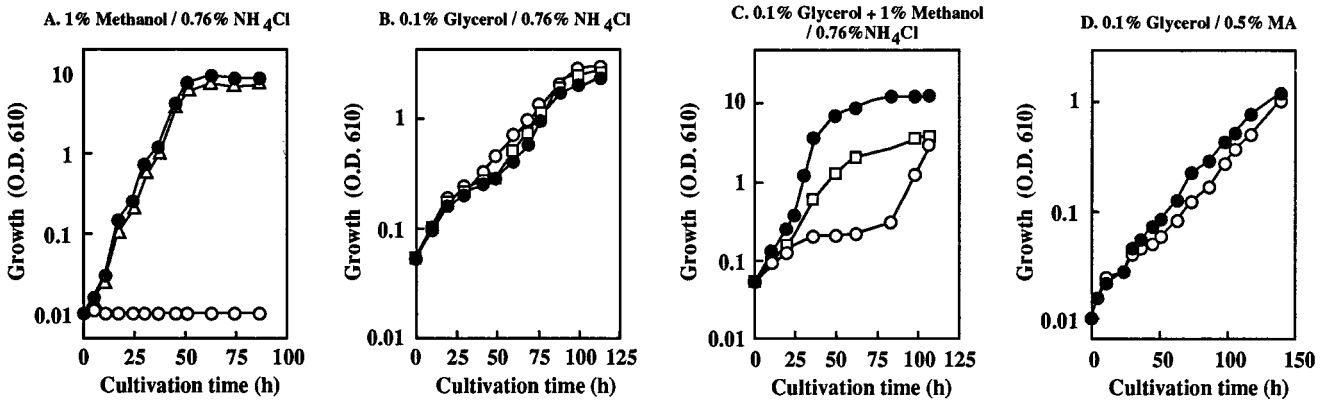

FIG. 4. Growth of the wild-type and *das1*Δ strains on various carbon and nitrogen sources. MA, methylamine. Symbols: ●, wild-type strain; ○, *das1*Δ strain; □, *aod1*Δ · *das1*Δ strain; △, *das1*Δ strain expressing *DAS1* under control of the *AOD1* promoter. O.D. 610, optical density at 610 nm.

both in a batch culture (Fig. 4A) and in a methanol-limited chemostat culture ($D = 0.05\ h^{-1}$). Again, these results differed from the growth of the *fdh1*Δ strain, which was retarded in a methanol batch culture and one-fourth the maximum yield in a methanol-limited chemostat culture (23). The growth of the *das1*Δ strain on methanol was restored by the expression of the coding region of the *DAS1* gene under control of the *C. boidinii AOD1* promoter (Fig. 4A).

Growth of the *das1*Δ strain had a prolonged lag period on medium containing glycerol plus methanol (Fig. 4C) relative to the rate for the wild-type strain. This growth inhibition may be due mainly to the toxicity of formaldehyde produced by AOD from methanol in the medium, since this growth retardation was not observed in the *das1*Δ *aod1*Δ strain, the double disruptant of *AOD1* and *DAS1* (Fig. 4C). In contrast to its growth on glycerol-plus-methanol medium, the *das1*Δ strain showed the same growth as the wild-type strain in media containing glycerol and methylamine (Fig. 4D) and glycerol and choline (data not shown).

Physiological role of DHAS as an assimilatory enzyme. The syntheses of methanol-assimilatory and -dissimilatory enzymes have been considered to be regulated under the same control system through methanol induction and glucose repression (14). In *C. boidinii*, the regulatory pattern of DHAS was similar to that of AOD. However, the regulation of AOD and DHAS was clearly distinct from the regulation of enzymes in the dissimilation pathway, i.e., FDH and FLD.

Our previous study of *FDH1* regulation and gene disruption revealed that the main physiological role of the glutathione-dependent formaldehyde oxidation pathway is detoxification of formaldehyde and formate (23). Comparison of the present results with those obtained in the previous study has revealed several differences in both regulation and knockout effect between the *DAS1* and *FDH1* genes. (i) The *fdh1*Δ strain retained the ability to grow on methanol, but the *das1*Δ strain did not. (ii) The *FDH1* expression was observed under all conditions where formaldehyde is generated in the cells, i.e., with media containing glucose and methylamine, glucose and choline, glycerol and methylamine, glycerol and choline, and glycerol plus methanol. However, *DAS1* expression was not detected in glucose-methylamine or glucose-choline medium. (iii) The defect in growth of the *fdh1*Δ strain was observed in all media where *FDH1* expression occurred. In contrast, even though *DAS1* was expressed in glycerol-methylamine and glycerol-choline media, we could not observe any defect in growth of the *das1*Δ strain on these media.

These results represent differences in the physiological roles of *DAS1* and *FDH1*: the main role of DHAS may be fixation of formaldehyde into cell constituents, which is different from that of FDH, which is involved in the detoxification of formate. This was supported by the observation that the wild-type strain had a growth yield ca. four times higher than those of the *das1*Δ strain or the *das1*Δ *aod1*Δ strain on medium containing glycerol plus methanol (Fig. 4C). Furthermore, the growth yields of these two *das1*Δ strains on medium containing methanol plus glycerol were the same as those on glycerol medium (Fig. 4B). These results indicate that methanol was not assimilated in these *das1*Δ strains during growth on medium containing glycerol plus methanol and strongly suggest that DHAS is involved mainly in the assimilation of formaldehyde.

In a previous study, we showed that Pmp47 is necessary for the translocation and folding of DHAS but not of AOD or CTA (26). Further analysis with the *DAS1* gene and the *das1*Δ strain obtained in this study will enable us to reveal the relationship between DHAS import into peroxisomes and the function of Pmp47 at the molecular level.

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