Volume 15 Number 9 1987 Nucleic Acids Research

Expression of the *lacZ* gene from two methanol-regulated promoters in *Pichia pastoris*

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Received January 22, 1987; Accepted April 3, 1987

ABSTRACT

Two DNA fragments containing putative control regions regulating the expression of the alcohol oxidase (AOX) and dihydroxyacetone synthase (DAS) genes from the methylotrophic yeast Pichia pastoris were used in the construction of vectors for the expression of the Escherichia coli lacZ gene. These vectors were transformed into P. pastoris host cells and employed in experiments to measure the control mechanisms employed by each promoter in the production of β-galactosidase fusion products. Results in P. pastoris suggest that the processes used to regulate the expression of these gene fusions involve both repression/derepression and induction mechanisms. Expression of the AOX-lacZ and DAS-lacZ fusions was examined in Saccharomyces cerevisiae as well. Interestingly, β-galactosidase was expressed in a regulated manner in the heterologous host.

INTRODUCTION

Metabolism of methanol by Pichia pastoris proceeds by a well-defined pathway (1,2). Alcohol oxidase (AO) functions as the first enzyme in this pathway, converting methanol to formaldehyde within the protective confines of yeast peroxisomes (see review, 3). We have previously reported the isolation and characterization of the AO gene, AOX, including the putative DNA sequences within the genome which regulate the expression of this gene (4). In addition, this report described the isolation and characterization of two other methanol-regulated genes. Subsequently, one of the two genes, P76, has been identified as encoding dihydroxyacetone synthase (DHAS), the first enzyme in the methanol assimilatory pathway, and its putative regulatory region has been defined (G. Thill, personal communication).

Studies on the mechanisms responsible for the regulation of several methanol-responsive genes have until recently been physiological in approach. Evidence for a repression/derepression

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mechanism has been derived from expression studies on the AOX (MOX) and catalase genes of Hansenula polymorpha and Kloeckera sp 2201 (5,6). The expression of these genes is repressed when cells are grown in the presence of glucose. Examples of glucose catabolite repression are well documented in yeasts such as those with glyoxylate cycle enzymes (7), the MAL6-locus (8), galactose utilization enzymes (9,10), invertase (11) and cytochrome C (12). However, increased levels of AO and catalase are detected if these cells are grown on glycerol [up to 70% of the AO activity of methanol-grown cells (13)], even though glycerol is not dependent on AO for its metabolism (14). Another related method for the regulation of the AOX gene in H. polymorpha and Kloeckera sp 2201 has been observed when these cells are grown on decreasing levels of glucose (5). This condition approaches carbon starvation and results in relieving the catabolite repression caused by glucose. Finally, induction of the AOX gene has been reported by Eggeling and Sahm as a second mechanism of regulation for this gene in H. polymorpha (14). However, in these and other experiments (15, 16), it appears unclear whether the effect observed is due to the ability of methanol to induce expression or is the result of derepression caused by the growth conditions of the cells.

Several questions concerning the regulation and relative strengths of the putative control regions from the P. pastoris AOX and DAS genes can be approached by forming gene fusions between these putative control regions and the E. coli β-galactosidase gene (17). Of interest are the questions: 1) Do the DNA segments previously identified indeed act as promoters and regulatory regions? 2) What are the comparative strengths of these two regulatory regions? and 3) What mechanism best describes the regulation of these putative promoters?

To approach an answer to each of these questions, vectors were constructed in which the AOX or DAS putative regulatory regions were used to control the expression of β-galactosidase. Experiments using these constructions have been performed with both P. pastoris and S. cerevisiae as host cells. The results of these experiments provide a measure of the utility of these constructions as expression vectors and also some understanding of

the mechanisms by which these promoters regulate their natural gene products.

MATERIALS AND METHODS

Strains and Media

E. coli strain MC1061 [F⁻araD139 Δ(araABDIC-leu) 7679 ΔlacX74 galU galK rpsL hsdR] was provided by M. Casadaban. Biochemical studies were done with the Saccharomyces cerevisiae strain SEY2101 (Matα leu2-3 leu2-112 ura3-52 his4-519 suc2Δ9) (18) and the Pichia pastoris strain GS115 (his4) (19).

E. coli were grown in LB medium and ampicillin was added when required to a final concentration of 100 μg/ml (20). Yeast were grown in yeast extract and peptone (YEP) or in yeast nitrogen base (without amino acids) (YNB) medium supplemented with the indicated carbon source (21). Yeast X-gal plates containing 0.1% glucose were prepared as described by Rose et al. (22).

Transformation and Plasmid Analysis

Transformations of E. coli (23), Saccharomyces cerevisiae (24), and Pichia pastoris (19) with plasmid DNA were done as described previously. To direct integration of vectors into the genome of P. pastoris, vectors pSAOH5 (Fig. 1) and pT76H4 (Fig. 2) were linearized with StuI prior to transformation. The restriction site StuI is located within the P. pastoris HIS4 gene and is uniquely present in both vectors. Transformants were analyzed for single integrants by preparation of total genomic DNA (19) and analyzed by Southern hybridization (25). Small-scale preparation of plasmid DNA from E. coli was done by an alkaline sodium dodecylsulfate method (23). Plasmids used for vector construction and subcloning are summarized in Table 1. Vector pBPf1, a gift from K. Barringer, was constructed as follows. Vector pYJ30 (19) was cut with BamHI and termini were filled in and ligated to yield vector pYM10 lacking a BamHI site. This vector was cut with EcoRI and PstI. The HIS4-containing fragment was ligated with the lacZ-containing fragment of pSEY101 (18), previously cut with PstI and NruI, to yield pBPf1. Restriction endonuclease and exonuclease digestions were done under conditions recommended by the suppliers, and modifications of DNA molecules with T4 ligase and polynucleotide kinase were performed as described by Maniatis et al. (23). DNA digests were analyzed

TABLE 1
PLASMIDS AND VECTORS USED FOR
CONSTRUCTION AND EXPRESSION STUDIES

Vectors	Hosts[1]	Contains Regulatory Region	Reference
1. pPG4.0	E. c.	AOX	Ellis et al., 1985
2. pSEY101	E. c.-S. c.	-	Douglas et al., 1984
3. pYJ30	E. c.-P. p.	-	Cregg et al., 1985
4. pSAOH5	E. c.-P. p.	AOX	This study
5. pTAO13	E. c.-S. c.	AOX	This study
6. pTAO11	E. c.-S. c.	AOX	This study
7. pPG6.0	E. c.	DAS	Ellis et al., 1985
8. pT76U1	E. c.-S. c.	DAS	This study
9. PT76H4	E. c.-P. p.	DAS	This study
10. pBPf1	E. c.-P. p.	-	This study

[1]E.c. = E. coli; S.c. = S. cerevisiae; P.p. = P. pastoris

by electrophoresis in 50 mM Tris/borate (pH 8.3) and 2 mM EDTA on 1% agarose gels (26). Restriction fragments were purified by electroelution from agarose gels into Whatman 3MM paper strips backed by dialysis tubing as described previously (27).

Growth of Yeast Cultures, Preparation of Cell-Free Extracts, and Enzyme Assays

Cells were initially grown in YNB medium containing 5% glucose at 30°C for 20 hours (mid-log phase, 1-2 OD_{600}). Samples were taken to determine the influence of glucose on the regulated expression of the lacZ gene. The remainder of the culture was collected by centrifugation in a table-top centrifuge (3000 RPM, 5 minutes) and transferred to a YNB medium (at final OD_{600} of 0.1-0.3) without glucose. These cultures were grown without a utilizable carbon source at 30°C for 20-30 hours to examine the effects of carbon starvation on lacZ expression. Finally, cells were collected by centrifugation and P. pastoris cultures were transferred to YNB medium (at a final OD_{600} of 0.1-0.3) containing 0.5% methanol. S. cerevisiae cells were transferred to YEP medium containing 3% glycerol as the final carbon source.

Cell-free extracts were prepared by withdrawing 20-50 OD_{600} units of cells (ca. 1 to 3 x 10^9 cells) from the culture medium. These cells were collected by centrifugation in a table-top centrifuge and washed with 1 ml cold lysis buffer (62.5 mM Tris•HCl,

pH 8.7, 5 mM EDTA, 2 mM PMSF). Thirty to fifty μl cold lysis buffer and 0.3-0.4 g of glass beads (0.45-0.5 mm diameter, B. Braun Melsungen AG) were added to the cell pellet. The suspension was then shaken 4 times at maximum speed on a vortex mixer for 1 minute, with one-minute intervals of cooling on ice. The cell slurry was removed, and the glass beads were washed with 30-50 μl of lysis buffer, which was combined with the cell slurry. This total cell extract was centrifuged in an Eppendorf centrifuge for 5 minutes. The supernatant (cell-free extract) was removed, and the membrane pellet was resuspended in an equal volume of lysis buffer (60-100 μl).

β-galactosidase assays were performed as described previously by Miller (20). A unit of β-galactosidase represents 1 nmole of o-nitrophenol produced per minute at 30°C and pH 7. The alcohol oxidase assay was performed as described previously (4). A unit of alcohol oxidase represents 1 nmole of H_2O_2 produced per minute at 30°C. AO assays, with methanol omitted, were run as blanks.

Immunodetection of β-galactosidase Fusion Protein by Western Hybridization

Pichia pastoris GS115 cells carrying plasmids pSAOH5 and pT76H4 were grown in YNB with glucose, no carbon source, or with methanol, as described above, and cell-free extracts were prepared from 20-50 OD_{600} units of cells. SDS gel electrophoresis was done according to the procedure of Laemmli (28). Western blot analysis of fusion proteins was done by the procedure of Towbin *et al*. (29) using β-galactosidase antiserum (Cooper Laboratories) and affinity-purified alcohol oxidase antiserum. The antigen-antibody complex was detected with 0.2 μCi ^{125}I-labeled protein A per lane (New England Nuclear; specific activity 8 μCi/μg).

Purification of *P. pastoris* alcohol oxidase was performed as described previously (4), with the exception that an additional DEAE ion exchange chromatography step was added. Alcohol oxidase was eluted at 0.24 M NaCl. Rabbit anti-alcohol oxidase protein antibodies were purified from whole antisera by chromatography through a CNBr-coupled alcohol oxidase Sepharose 4B column. Protein was estimated by the method of Lowry *et al*. (30) using BSA as a standard.

RESULTS

Construction of AOX- and DAS-lacZ Gene Fusions

In order to investigate regulation of expression of P. pastoris alcohol oxidase (AOX) and dihydroxyacetone synthase (DAS) genes, the putative promoter region from each of these genes was fused to the lacZ gene from E. coli. Figure 1 illustrates the

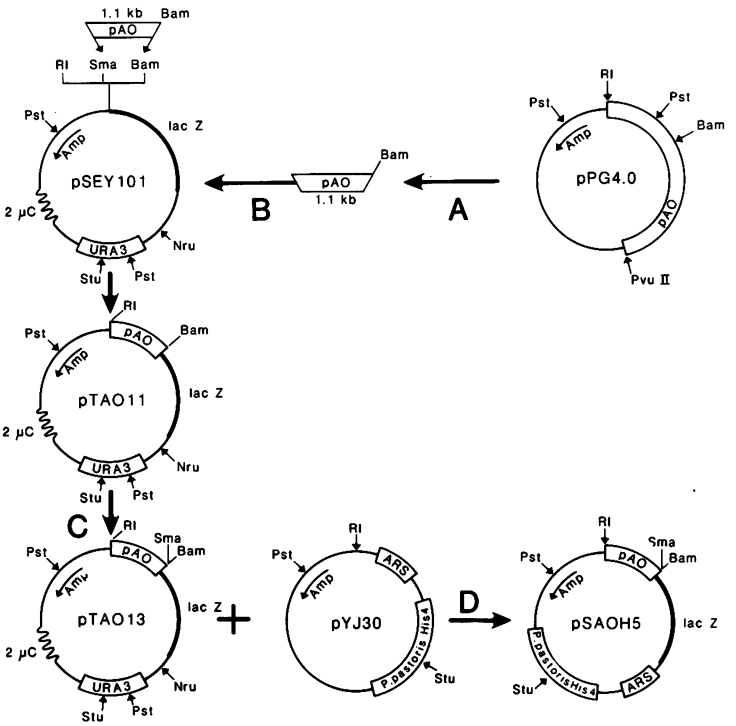

Figure 1. Construction of AOX-lacZ Gene Fusion. The plasmid pPG4.0 contains a copy of the alcohol oxidase gene from the genome of P. pastoris. The AOX regulatory region is encoded on an 1100 base pair BamHI-PstI fragment of this plasmid. pPG4.0 was cleaved with PstI, treated with S1 nuclease to produce a blunt-ended fragment, and subsequently digested with BamHI (A). Insertion of this regulatory region into the E. coli-S. cerevisiae shuttle vector pSEY101 at the 5′ end of the E. coli lacZ gene produced pTAO11 (B). An in-frame fusion with the first 15 amino acids of the AOX gene and the lacZ gene (starting at the ninth amino acid) was made by the insertion of a synthetic oligonucleotide (5′-GATCACCCGGGT-3′) at the BamHI site of pTAO11 to produce pTAO12 (not shown). The AOX regulatory region of pTAO12 was ligated as an EcoRI-SmaI fragment into the EcoRI-SmaI sites of pSEY101 to yield pTAO13 (C). The larger PstI-NruI fragment of pTAO13 was ligated with the HIS4-containing fragment of vector pYJ30, previously cut with EcoRI and PstI, to produce pSAOH5 (D).

construction of plasmids pSAOH5 and pTAO13, both expressing β-galactosidase using the putative alcohol oxidase promoter as a regulatory element. Plasmid pSAOH5 contains the P. pastoris HIS4 gene (a selectable marker), the AOX-lacZ fusion, and a 164 base pair TaqI fragment, PARS1, possessing P. pastoris autonomous replicating sequence (ARS) properties (19). The 2μ circle-based plasmid pTAO13 was constructed to study expression of the AOX-lacZ fusion in a ura3 auxotroph of a Saccharomyces cerevisiae host. Figure 2 illustrates the construction of plasmids pT76H4 and pT76U1, expressing β-galactosidase using the putative control

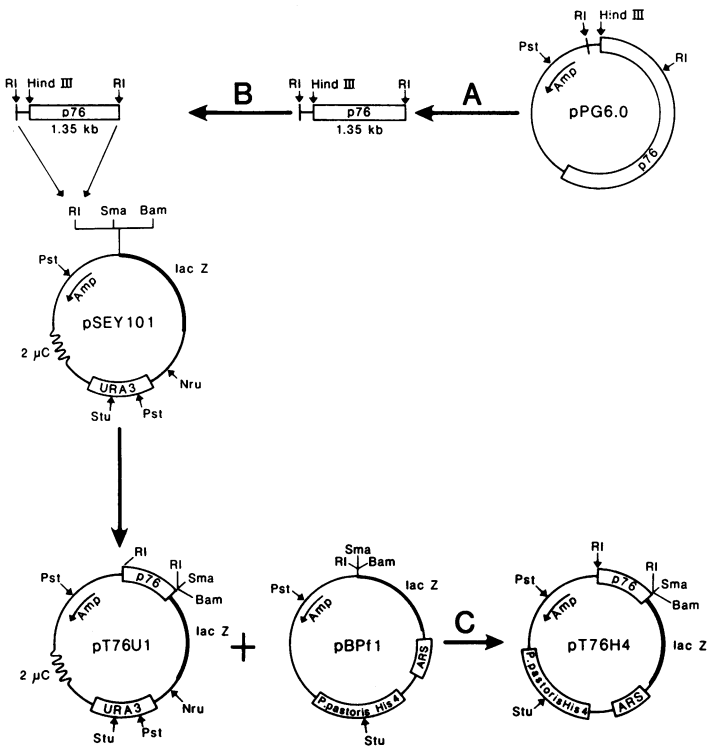

Figure 2. Construction of DAS-lacZ Gene Fusion. The plasmid pPG6.0 contains a copy of the DAS gene from the genome of P. pastoris. The DAS regulatory region is encoded on a 1350 base pair HindIII-EcoRI fragment of this plasmid (A). Insertion of this regulatory region, obtained by cleaving pPG6.0 with EcoRI, into the EcoRI site of pSEY101 produced pT76U1 (B). The PstI-BamHI fragment in pBPf1 was replaced by the PstI-BamHI fragment of pT76U1 to produce plasmid pT76H4 (C).

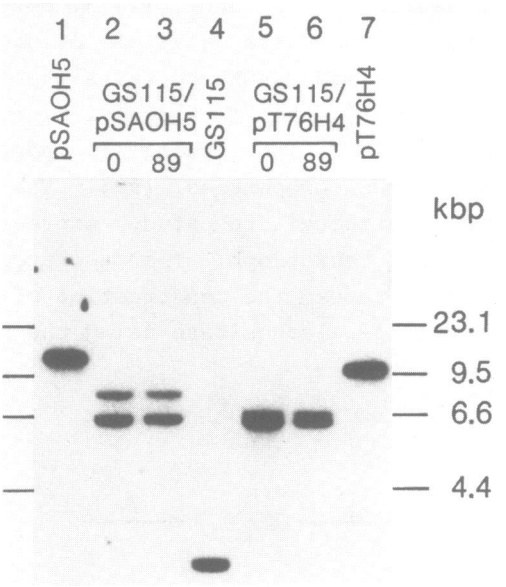

Figure 3. Southern blot hybridization of yeast cellular DNAs with ^{32}P-labeled P. pastoris HIS4 gene. Lanes 1 and 7 each contain 5 ng of EcoRI-restricted pSAOH5 and pT76H4, respectively, from E. coli cells. Lanes 2, 3, 5, and 6 contain 3 μg of EcoRI-restricted DNA from pSAOH5- or pT76H4-transformed GS115 cells before (0 hrs.) and after (89 hrs.) growth in glucose and methanol. Lane 4 contains 3 μg of EcoRI-restricted DNA from GS115 cells.

region of the P. pastoris DAS gene. Similar to pSAOH5, plasmid pT76H4 contains the P. pastoris HIS4 for selection and can either be maintained as an autonomous element or integrated into the P. pastoris genome. The 2μ circle-based plasmid pT76U1 was constructed to study expression of the DAS-lacZ fusion in a ura3 auxotroph of an S. cerevisiae host.

Vectors pSAOH5 and pT76H4 were directed into the HIS4 gene of the P. pastoris host to ensure that stable expression of single-copy integrants was achieved for this study. Figure 3 presents a Southern blot in which the plasmids pSAOH5 and pT76H4 and total genomic DNA cleaved with EcoRI were probed with a ^{32}P-labeled DNA fragment containing the P. pastoris HIS4 gene. As predicted from the maps presented in Figures 1 and 2, a single fragment is observed in lanes containing EcoRI-digested pSAOH5 and pT76H4. The lanes containing EcoRI-digested total DNA from

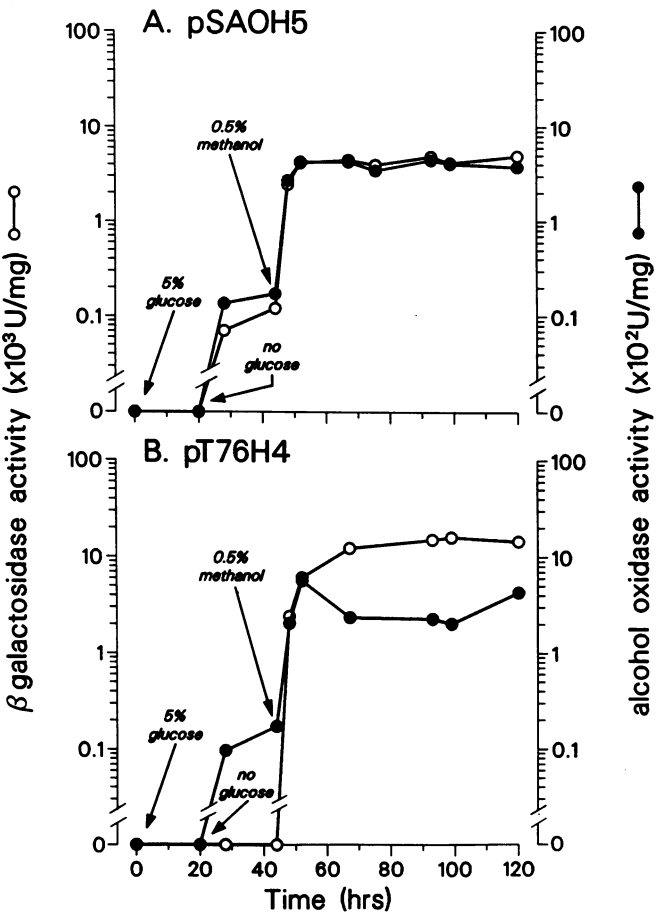

Figure 4. The effects of various carbon sources on the regulated expression of fusion genes and alcohol oxidase. The influence of glucose (5%), methanol (0.5%), and carbon starvation on P. pastoris cells carrying either pSAOH5 (A) or pT76H4 (B) were studied by measuring units (U) of β-galactosidase and alcohol oxidase activity per mg of soluble extracted protein.

pSAOH5- and pT76H4-containing cells reveal two copies of the HIS4 fragment. A single copy of each integrated expression vector was present in the genome of each host before (0 hours) and after growth of the cells in glucose and methanol (89 hours). The untransformed host cell GS115 possesses a HIS4-containing fragment which is small relative to that of strains containing the integrated expression plasmids.

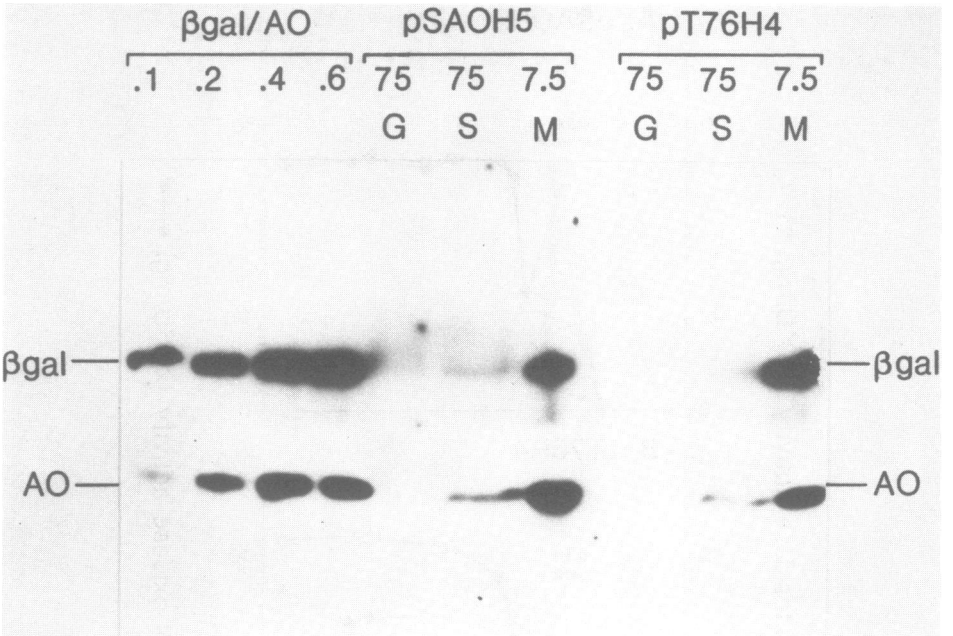

Figure 5. Detection of AOX-lacZ and DAS-lacZ fusion proteins and alcohol oxidase in cell-free extracts of P. pastoris by Western hybridizations. Cells containing pSAOH5 and pT76H4 were grown in YNB medium and 5% glucose (G), under carbon starvation (S), or 0.5% methanol (M) at 30°C. The culture under carbon starvation was maintained in stationary phase for 24 hours. A total of 7.5 or 75 μg of cell-free protein extract was loaded on each lane for electrophoresis and Western hybridization. 0.1 to 0.6 μg of purified E. coli β-galactosidase and P. pastoris alcohol oxidase served as a standard.

Effects of Carbon Sources on the Regulated Expression of Gene Fusions

Expression in P. pastoris. The regulation of the AOX and DAS promoters controlling expression of the lacZ fusions was examined by measuring the levels of β-galactosidase in cells which were grown in the presence of a repressing or inducing carbon source. In P. pastoris, glucose has been observed to be a repressor of alcohol oxidase activity (4,13,31,32). The cells were initially grown to mid-log phase in 5% glucose and then switched to fresh medium without glucose at a final OD_{600} unit of 0.2-0.5 (Fig. 4). The levels of β-galactosidase expression were followed in cells carrying either pSAOH5 or pT76H4. P. pastoris

cells not carrying the lacZ fusion gene constructs do not express β-galactosidase (data not shown). After 25 hours, with no detectable glucose present in the growth medium, an increase in the levels of both β-galactosidase (from 1 to 120 U/mg) and alcohol oxidase (from 0 to 17 U/mg) activities in cells carrying pSAOH5 was detected (Fig. 4A). In cells carrying pT76H4, grown under identical conditions, a similar increase of alcohol oxidase activity (from 0 to 17 U/mg) was measured (Fig. 4B), but no increase in β-galactosidase activity was observed. The addition of methanol caused a further increase in β-galactosidase (usually to a maximum of 5 to 13 x 10^3 units/mg of protein) and alcohol oxidase activities (5 x 10^2 units/mg of protein) within 20 hours in cells containing pSAOH5. The introduction of methanol to cells carrying pT76H4 caused a dramatic increase in the levels of β-galactosidase activity (usually from 1 to a maximum of 10 to 20 x 10^3 units/mg of protein) and a similar increase of alcohol oxidase activity, as observed in cells containing pSAOH5 (Fig. 4B). These increases in enzyme activities, observed during carbon starvation and after growth on methanol, are substantiated by increases in β-galactosidase and alcohol oxidase proteins as detected by Western blot analysis (Fig. 5). Greater than 90% of total alcohol oxidase and β-galactosidase was present in the cell-free extract of the P. pastoris cells carrying either pSAOH5 or pT76H4 (data not shown).

Vectors pSAOH5 and pT76H4 were also used for transformation of the P. pastoris host GS115, without previous linearization with restriction enzyme StuI. However, these autonomous vectors appeared to be unstable during growth after several generations, even in medium lacking histidine. In an experiment similar to the one shown in Figure 4, but with autonomous replicating vectors pSAOH5 and pT76H4, qualitatively the same result was found, except that the absolute β-galactosidase levels differed. During the starvation phase, the autonomous vector pSAOH5 produced 10- to 20-fold higher β-galactosidase activity levels than its integrated counterpart. After the initial shift in methanol, β-galactosidase levels produced by both autonomous vectors pSAOH5 and pT76H4 occasionally increased up to 2- to 3-fold above the levels found for the integrated vectors. Further growth in meth-

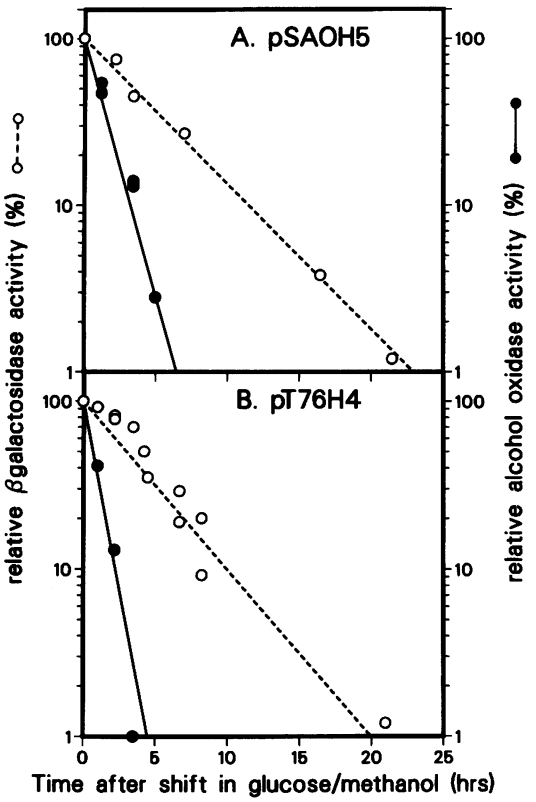

Figure 6. The effects of glucose on the expression of AOX-lacZ and DAS-lacZ gene fusions. P. pastoris cells cultured in the presence of 0.5% methanol were switched to 0.5% methanol and 2% glucose. Cells containing pSAOH5 (A) and pT76H4 (B) were measured for β-galactosidase (o-o) and alcohol oxidase (●-●) activities after the switch to glucose and methanol. Enzyme activities were measured in units per mg of cell-free protein extract at each time-point and are indicated in relative activity units.

anol was accompanied by a decrease in these levels. Differences in expression levels seen between integrated and autonomous vectors are thought to be due to the effect of multiple copy numbers and subsequent plasmid loss or plasmid integration (data not shown).

Repression of the AOX and DAS promoters by the use of glucose as a carbon source was further demonstrated by growing cells first in the presence of methanol (0.5%) until significant levels of β-galactosidase (5 to 10 x 10^3 units/mg) had been reached

TABLE 2
MAXIMUM LEVELS OF β-GALACTOSIDASE ACTIVITY IN SACCHAROMYCES CEREVISIAE

Plasmid	Carbon Source	Enzyme Activity[1]
pTA013	5% glucose	14
pT76U1	5% glucose	10
pTA013	none	40
pT76U1	none	68
pTA013	3% glycerol	950
pT76U1	3% glycerol	3210
pLG669Z	3% raffinose	3600
pSEY125	0.1% glucose	150
pGP-Z1	2% galactose	1300

[1]β-galacotsidase activity in units per milligram protein of total cell extract.

(Fig. 6). These cells were then switched to a medium containing 2.0% glucose and 0.5% methanol, and the levels of β-galactosidase and alcohol oxidase activities were monitored. Immediately after the shift, cells containing either pSAOH5 or pT76H4 demonstrated a rapid reduction in the activity of both alcohol oxidase and β-galactosidase (Fig. 6). The more rapid decline of alcohol oxidase activity relative to either of the fusion products will be addressed in the discussion.

Expression in Saccharomyces cerevisiae. The plasmids pTAO13, containing the AOX-lacZ fusion, and pT76U1, containing the DAS-lacZ fusion, were used to determine whether expression of these fusions could be observed in S. cerevisiae and whether expression of the fusions was regulated in this heterologous host. Expression of the fusion proteins from the multi-copy, autonomous 2μ circle-based plasmids pTAO13 and pT76U1 in S. cerevisiae grown on 5% glucose achieved levels of only 14 and 10 units/mg protein, respectively (Table 2). After 20 hours of growth, the cells were transferred to media lacking glucose. The carbon starvation conditions apparently resulted in a derepression of β-galactosidase activity in cells containing either the AOX-lacZ or DAS-lacZ fusion. Glycerol was added to the cultures at a final concentration of 3%, resulting in a further increase of β-galactosidase activity for both pTAO13- and pT76U1-containing cells. Two μ circle-based lacZ gene fusion vectors with the

cytochrome C promoter [pLG669Z (33)], the invertase promoter [pSEY124 (34)], and the galactose permease promoter [pGP-Z1 (35)] were used to compare lacZ expression levels in S. cerevisiae (Table 2).

DISCUSSION

In this paper we present the first description of methanol-regulated expression of a heterologous gene. All available evidence suggests that the two DNA fragments described in this report contain the regulatory regions of the AOX and DAS genes of P. pastoris. The 1.1 kilobase pair PstI-BamHI DNA fragment preceding the alcohol oxidase gene and the 1.35 kilobase pair EcoRI fragment preceding the dihydroxyacetone synthase gene are capable of providing regulated expression of a heterologous gene (lacZ) in cells of Pichia pastoris. Figure 4 illustrates the capability of both these DNA fragments to respond in an appropriate fashion. Upon shifting the AOX-lacZ-containing cells from a repressing condition (glucose) to a derepressing condition (carbon starvation), both alcohol oxidase and β-galactosidase activities increase significantly. Levels of β-galactosidase reach 2-4% of the maximal levels observed in methanol. A further large increase in both enzyme activities is observed after shifting the cells to methanol medium.

A significant difference between expression of AOX and the AOX-lacZ fusion appears to be the quantity of protein synthesized. From the immmunoblots (Fig. 5), we estimate that there is 5 to 10 times more AO protein than β-galactosidase protein in both carbon-starved and methanol-grown cells. The low level of β-galactosidase is not the result of a position effect, since expression levels in P. pastoris strains which contain pSAOH5 integrated at the AOX promoter locus (a different integration site) are indistinguishable from those reported here (data not shown). Lower yields of heterologous gene products relative to those from the native gene are routinely observed in S. cerevisiae and are ascribed to lower stability or translatability of the heterologous mRNAs or to the absence of transcriptional activation sequences downstream of the promoter fragments (36,37). However, lower yields of heterologous proteins in P. pastoris

relative to alcohol oxidase may simply be explained by a higher turnover rate of cytoplasmic versus peroxisomal proteins. In our studies, the ratio of alcohol oxidase and β-galactosidase activity and protein under each growth condition remains approximately the same. Thus, we believe that the putative AOX regulatory fragment is regulating expression of lacZ in the proper manner.

The regulation of lacZ expression under control of the DNA fragment preceding the DAS gene is consistent with what is known about the synthesis of dihydroxyacetone synthase in P. pastoris and other methylotrophic yeasts. The DAS-lacZ fusion is strongly repressed in glucose-grown cells, but is expressed at high levels in methanol-grown cells (3,4,31). Expression of the DAS-lacZ fusion in P. pastoris does not occur under derepressing growth conditions, which was also observed for the DAS gene in H. polymorpha (31).

Both the AOX and DAS regulatory regions are repressed when P. pastoris cells carrying these constructions are grown on a mixture of glucose and methanol. Glucose repression leads to a virtual shut-down of expression from these regulatory regions. The rapid rate at which AO activity is reduced relative to that of either of the fusion products may be a consequence of the peroxisomal location of AO. Upon shifting from methanol to repressing growth conditions, peroxisomes are known to fuse with vacuoles, leading to the active degradation of enzymes compartmentalized in this organelle (3). Since the AOX-lacZ fusion protein is not translocated into peroxisomes (data not shown), it seems likely that the fusion protein is not subject to this rapid degradation process. The small contribution of 4 amino acids of DHAS to the DAS-lacZ fusion product (G. Thill, personal communication) makes it unlikely that this fusion product is translocated into peroxisomes either.

Although expression of these lacZ fusions from these two regulatory regions is similar in most respects, there is at least one obvious difference. While the AOX control region is activated in response to carbon starvation, the DAS control region is not. The observation that high levels of AOX and DAS promoter-driven expression are achieved only in response to methanol suggests that the regulatory mechanism controlling these genes

involves an induction component responding to methanol or a metabolite of methanol. Further studies are in progress to investigate the possible presence of positive and negative regulatory factors similar to those described for the regulation of the GAL1, GAL7, and GAL10 gene cluster (38-41).

Comparison of the results of the biochemical studies on H. polymorpha and the lacZ expression studies on P. pastoris suggests differences between the regulatory mechanisms of methanol-pathway genes in these two methylotrophic yeasts. Since high levels of methanol-metabolizing enzymes in H. polymorpha are also observed with glycerol as a carbon source (14), their regulation best fits the description of a repression/derepression mechanism. In contrast, no expression of AOX, or the AOX-lacZ or DAS-lacZ fusions, is observed in glycerol-grown cells of P. pastoris (data not shown), and only a relatively small derepression response is observed upon carbon starvation compared to fully induced cells in methanol. In P. pastoris, methanol is the only carbon source we have found which is able to elicit high levels of methanol biosynthetic-pathway enzymes. It will be interesting to learn whether the regulatory differences between these two methylotrophs reflect only a quantitative difference in otherwise very similar regulatory mechanisms or the existence of fundamentally different types of control mechanisms in these yeasts.

We have examined both the AOX-lacZ and the DAS-lacZ gene fusions in the heterologous host S. cerevisiae and have found that both P. pastoris regulatory regions promote expression of a protein with β-galactosidase activity. The gene fusions synthesized as much β-galactosidase activity as those from S. cerevisiae GAL2- or CYC1-regulated sequences. The observed regulation of β-galactosidase activity in S. cerevisiae suggests that these P. pastoris DNA sequences function in a specific way in the heterologous host. However, it remains to be determined whether the expression of β-galactosidase activity in S. cerevisiae depends on the same DNA sequences specific for regulation of the AOX and DAS genes in its natural host, P. pastoris.

ACKNOWLEDGEMENTS

We thank R. Buckholz, G. Davis, M. E. Digan, M. M. Harpold and G. Thill for their helpful discussions and comments. We also thank D. Stroman and J. Cruze of the Phillips Petroleum Company

for their discussion and assistance throughout this project. We wish also to acknowledge J. Doty and S. Konchal for their help in the preparation of this manuscript. This research was supported by Phillips Petroleum Company.

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