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Mxr1p, a Key Regulator of the Methanol Utilization Pathway and Peroxisomal Genes in *Pichia pastoris*

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Growth of the yeast *Pichia pastoris* on methanol induces the expression of genes whose products are required for its metabolism. Three of the methanol pathway enzymes are located in an organelle called the peroxisome. As a result, both methanol pathway enzymes and proteins involved in peroxisome biogenesis (PEX proteins) are induced in response to this substrate. The most highly regulated of these genes is *AOX1*, which encodes alcohol oxidase, the first enzyme of the methanol pathway, and a peroxisomal enzyme. To elucidate the molecular mechanisms responsible for methanol regulation, we identify genes required for the expression of *AOX1*. Mutations in one gene, named *MXR1* (methanol expression regulator 1), result in strains that are unable to (i) grow on the peroxisomal substrates methanol and oleic acid, (ii) induce the transcription of *AOX1* and other methanol pathway and *PEX* genes, and (iii) form normal-appearing peroxisomes in response to methanol. *MXR1* encodes a large protein with a zinc finger DNA-binding domain near its N terminus that has similarity to *Saccharomyces cerevisiae* Adr1p. In addition, Mxr1p is localized to the nucleus in cells grown on methanol or other gluconeogenic substrates. Finally, Mxr1p specifically binds to sequences upstream of *AOX1*. We conclude that Mxr1p is a transcription factor that is necessary for the activation of many genes in response to methanol. We propose that *MXR1* is the *P. pastoris* homologue of *S. cerevisiae ADR1* but that it has gained new functions and lost others through evolution as a result of changes in the spectrum of genes that it controls.

The ability to utilize methanol as a carbon and energy source is limited in eukaryotes to a few yeast species (1, 34, 57). The metabolic pathway is nearly identical in each species and begins with the oxidation of methanol to formaldehyde, which is catalyzed by the peroxisomal matrix enzyme alcohol oxidase (Aox). A by-product of this reaction is hydrogen peroxide, which is subsequently degraded to water and oxygen by a second peroxisomal enzyme catalase (Cat). The formaldehyde generated by Aox follows one of two paths. A portion leaves the peroxisome and is further oxidized by two cytoplasmic enzymes, formaldehyde dehydrogenase (Fld) and formate dehydrogenase (Fdh), to generate energy for the cell. The remaining formaldehyde is condensed with xylulose-5-phosphate by a third peroxisomal enzyme, dihydroxyacetone synthase (Dhas), to generate two three-carbon molecules that leave the peroxisome and enter a cyclic pathway that regenerates xylulose-5-phosphate and produces one net molecule of glyceraldehyde-3-phosphate for every three turns of this cycle (1, 57).

Because three of the methanol pathway enzymes (Aox, Cat, and Dhas) are peroxisomal, the function of this organelle is also essential for methanol growth (21, 26, 33). This observation has made *Pichia pastoris* a major model system for the elucidation of peroxisome biogenesis and function (2, 40, 49). One advantage of *P. pastoris* for peroxisome studies is that in addition to methanol utilization, the yeast harbors a second peroxisomal metabolic pathway, a β-oxidation system, which permits growth on fatty acids such as oleic acid. Virtually all mutants of *P. pastoris* that are simultaneously and specifically defective in methanol and oleate growth (but normal for growth on other carbon sources) are affected in genes involved in peroxisome biogenesis (*PEX* genes) (26, 33). To date, approximately 20 *PEX* genes have been identified in this yeast (24, 39). These *PEX* genes encode proteins (called peroxins or PEX proteins), many of which appear to be components of the peroxisomal protein import machinery.

P. pastoris is best known as a popular system for the production of recombinant proteins (6). Over 550 such proteins have been synthesized in this yeast (http://faculty.kgi.edu/cregg/index.htm). Since most foreign genes expressed in *P. pastoris* are transcribed under the control of the promoter from the *P. pastoris AOX1* gene, transcription in response to methanol is a key feature of this expression system. However, little is known about how methanol regulates *AOX1* and other genes needed for methanol growth in *P. pastoris* or any other yeast. Previous studies by our laboratory and by others have shown that *AOX* protein and message are undetectable in cells grown on

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TABLE 1. *P. pastoris* strains used in this work

Strain	Genotype	Reference or source
JC100	Wild type	14
GS115	*his4*	14
JC132	*mxr1-1 his4*	26
JC133	*mxr1*Δ*::HIS4 his4*	This study
JC134	*mxr1*Δ*::HIS4 his4 ade1*	This study
JC135	*mxr1-1 his4 MXR1*	This study
JC300	*ade1 arg4 his4*	5

glucose but can constitute more than 30% of total cellular protein and 5% of the total poly(A)$^+$ mRNA in methanol-grown cultures (9, 11). Fusions of the *AOX1* 5′ region to reporter genes have demonstrated that this regulation occurs primarily at the transcriptional level and indicate the existence of both repression/derepression mechanisms responding to glucose and other carbon sources as well as a methanol-specific induction mechanism (11, 12, 53). Typically, derepressed (carbon-starved) cells display levels of *AOX1* transcriptional activity that are approximately 2% of that seen on methanol, whereas methanol-grown cells display activity that is more than 1,000-fold higher than that observed in fully repressed (glucose-grown) cells (53). In this respect, methanol regulation is similar to that of alternative carbon source pathways in many microorganisms (42). Interestingly, ethanol, which like methanol, is a small alcohol and a gluconeogenic carbon source and also strongly represses transcription of *AOX1* and other methanol pathway genes. This makes sense from a physiological perspective, since Aox, if present, would oxidize ethanol nearly as readily as methanol, which would result in the generation of large amounts of acetaldehyde in the peroxisome, a disaster for the cell. How the cellular regulatory machinery manages to distinguish between these similar compounds is a mystery.

The goal of our studies is to understand, at the molecular level, how *P. pastoris* coordinately regulates the expression of *AOX1* and other genes necessary for methanol utilization. As an initial step, we have identified and characterized a gene, *MXR1* (for methanol expression regulator 1), whose product, Mxr1p, is a *trans*-acting factor essential for significant levels of methanol pathway and *PEX* gene transcription in response to methanol. Mxr1p shows sequence similarity to certain DNA-binding transcription factors, and we show that the protein shifts in localization to the nucleus upon exposure of cells to methanol and specifically binds to *AOX1* 5′ promoter sequences. These results represent a first step in the elucidation of the regulatory circuits that control methanol metabolism in *P. pastoris*.

MATERIALS AND METHODS

Media, strains, and microbial techniques. *P. pastoris* strains used in this study are listed in Table 1. *P. pastoris* cells were cultured in either YPD medium (1% yeast extract, 2% peptone, 2% glucose) or minimal YNB medium (0.67% yeast nitrogen base without amino acids) supplemented with either 0.4% glucose (YND), 0.5% methanol (YNM), or 0.1% oleic acid and 0.5% Tween 40 (to solubilize the oleate) (YNO). Oleate-induced cultures that were used for differential centrifugation experiments were also supplemented with 0.05% yeast extract. Amino acids were added to 50 μg/ml to strains requiring support for auxotrophic growth. All growth of *P. pastoris* strains was done at 30°C. For *P. pastoris*, zeocin was added to a final concentration of 100 μg/ml for transformation unless otherwise noted (see below). Transformations of *P. pastoris* were done by using the spheroplast method (10), the *Pichia* EasyComp kit (Invitrogen, Carlsbad, CA), or the electrotransformation method (13). *P. pastoris* strain construction, which required the mating of haploid strains and sporulation of the resulting diploid products, was performed as previously described (14).

Escherichia coli cells, mainly DH5 and TOP10 (Invitrogen, Carlsbad, CA), were cultured in LB medium (0.5% yeast extract, 1% glucose, 0.5% NaCl) at 37°C for use with recombinant DNA techniques. Antibiotics were added to LB medium at the following final concentrations: ampicillin, 100 μg/ml; kanamycin, 40 μg/ml; and zeocin, 25 μg/ml for plasmid selection. Transformation and other standard recombinant DNA techniques used in this study for *E. coli* were performed as described previously (41).

Induction on carbon sources. For induction studies, cells were first grown overnight in YPD medium to stationary phase. Aliquots of the stationary culture were used to inoculate an appropriate volume of YND medium containing any nutritional supplements and grown to an optical density at 600 nm (OD_{600}) of approximately 0.5. An aliquot of these glucose-grown cells (approximately 25 OD_{600} units) was removed, and the cells were centrifuged, washed with water, and frozen away at −80°C. The remainder of the culture was centrifuged for 3 min at 2,000 × *g*, and the cell pellet, after being washed with 1 ml of water, was suspended in prewarmed YNM or YNO medium containing any necessary supplements. The cells were grown with vigorous shaking at 30°C for 8 h for induction, and cell pellets were harvested by centrifugation as described above. Cell pellets were then processed for protein or RNA extraction.

Cell fractionation, enzyme assays, and Western analysis. Crude extracts and subcellular fractionations were prepared according to procedures described previously (33). Enzyme assays for peroxisomal catalase (20), alcohol oxidase (55), acyl coenzyme A (CoA) oxidase (17), and mitochondrial cytochrome *c* oxidase (18) were done at 30°C using standard protocols. Assays for β-lactamase (58), β-galactosidase (41), and glyceraldehyde-3-phosphatase (58) were performed at room temperature using standard protocols. Assays for formaldehyde dehydrogenase and formate dehydrogenase have been described previously (43). For differential centrifugation experiments, Western blots, and enzyme assays, protein concentrations were determined using the Pierce (Rockford, IL) bicinchoninic acid protein assay kit with bovine serum albumin as a standard. Cytosolic and pellet fractions containing 25 μg of protein from differential centrifugations as well as total cellular protein from extracts were loaded onto stacked sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels for electrophoresis (29). Proteins were then transferred onto nitrocellulose using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. Immunoblots were done using polyclonal antibodies to the antigen and were visualized with the Western-Light protein detection kit (Tropix, Bedford, MA). The anti-*myc* antibody was purchased from Invitrogen (Carlsbad, CA); all other antibodies were produced in our laboratory.

Isolation of plasmid pYL1. Plasmid pYL1 was isolated from a *P. pastoris* genomic DNA library using the Sib selection procedure (3, 31). The library was constructed from an *E. coli-P. pastoris* shuttle vector (pYM8) with fragments of *P. pastoris* genomic DNA inserted at a unique BamHI site in the vector (32). pYM8 contains the *Saccharomyces cerevisiae HIS4* gene for selection in *P. pastoris* and an ampicillin resistance gene for selection in *E. coli*. The library was transformed into *E. coli* MC1061, and cells were spread onto 10 150- by 15-mm LB plates supplemented with ampicillin (LB-Amp). Each of these master plates contained 1,000 to 2,000 transformed colonies which were then replica plated onto LB-Amp. The replica plates were incubated overnight at 37°C, and the colonies from each plate were collected by washing the plate with 15 ml of LB-Amp. Cells were inoculated into 200-ml liquid cultures of LB-Amp and incubated at 37°C overnight. Plasmid DNA was purified from each of these cultures using a QIAprep Spin Miniprep kit (QIAGEN, Chatsworth, CA). This DNA (approximately 1 to 2 μg) was then used to individually transform 10 sets of *P. pastoris* JC132 cells using the spheroplast method (13). Transformed yeast cells were first grown on YND plates to select for His$^+$ prototrophy and then replica plated onto YNM medium to further select those cells that were also Mut$^+$ (methanol utilization positive). Two of the 10 sets of JC132 transformants yielded Mut$^+$ colonies, indicating that the two sublibraries used to transform these sets were likely to contain at least one plasmid carrying a gene to correct the JC132 mutation. *E. coli* colonies from one master plate were picked and streaked onto fresh LB-Amp plates (100 streaks/plate). After streaks were grown overnight, each second-generation master plate was replica plated onto LB-Amp and grown overnight. The replica-plated colonies from each plate were collected by washing with 5 ml of LB-Amp, and plasmid DNA was prepared from each sample. These plasmid preparations were used to individually transform eight sets of *P. pastoris* JC132 cells by the spheroplast method, and transformants were

selected as described previously (13). Colonies from one *E. coli* master plate whose plasmid DNA yielded Mut$^+$ yeast transformants were picked and streaked onto LB-Amp plates in groups of 10. The process of replica plating, plasmid preparation, and transformation was repeated a third time. Finally, plasmid DNA was extracted from each of six individual *E. coli* strains and transformed into JC132. One plasmid, pYL1, transformed JC132 to Mut$^+$ at a high efficiency and contained a genomic insert of approximately 14 kb.

Subcloning of pYL1. In order to determine what portion of the *P. pastoris* DNA in pYL1 contained *MXR1*, regions of the 14-kb genomic insert were deleted by restriction digestion and self-ligation to create new plasmids. Three of these plasmids were made: pYL1Spe (pYL1 with a 7-kb SpeI fragment missing), pYL1Eag (pYL1 with a 0.9-kb EagI fragment missing), and pYL1Stu (pYL1 with a 2-kb StuI fragment missing). All plasmids were transformed into JC132 to determine which could still complement the mutant.

Knockout of *MXR1*. To disrupt *MXR1*, the 3.4-kb SacI-XhoI fragment of pYL1, which contained almost the entire *MXR1* coding sequence, was first subcloned into the same sites in the polylinker of pBluescript II SK(−) (Stratagene, La Jolla, CA) to generate pGC201. The *P. pastoris HIS4* gene was subcloned as a 2.7-kb BglII fragment from pYJ8ΔCla (10) and inserted into the BamHI site of pBluescript II SK(−) to create pGC111. The EagI-HindIII fragment was then excised from pGC201 and replaced with the EagI-HindIII fragment of pGC111, containing *P. pastoris HIS4*, to produce pGC202. The SacI-XhoI fragment of pGC202 was then used to transform GS115 by electroporation. The SacI-XhoI fragment contained *P. pastoris HIS4* flanked by regions of *MXR1* to direct homologous recombination. His$^+$ colonies were selected on YND plates and screened for the inability to grow on medium containing methanol as a sole carbon source. The Mut$^-$ strain was named JC133. Colony PCR, utilizing the primers MXRATG1 and HIS4C1, was used to confirm the disruption of *MXR1*, as described previously (5). JC134 (*mxr1*Δ*::HIS4 ade1*) was generated by crossing JC133 (*mxr1*Δ*::HIS4 his4*) with JC300 (*ade1 arg4 his4*), sporulating the resulting diploids, and then selecting the desired haploids. To prove that *MXR1*, and not an extragenic suppressor, was cloned, JC132 was crossed with JC134. All of the diploids as well as their consequential haploid spore products were Mut$^-$.

Promoter reporter studies. The construction of the promoter-reporter fusion vectors P_{AOX1}*-bla* (pHW018) and P_{GAP}*-bla* (pHW019) have been described previously (58). The P_{PEX8}*-bla* vector, pGC150, was created by excising the 735-bp *AOX1* promoter fragment from pHW018 with EcoRI and BglII and inserting an EcoRI-BglII fragment from pJS1 (a gift of Jay Sunga, Keck Graduate Institute, Claremont, CA) that contains the *PEX8* promoter. P_{MXR1}*-bla* was constructed by amplifying an approximately 700-bp promoter fragment from *MXR1* using the primers MXRP2 and MXRR1. The PCR product was digested with EcoRI and BamHI and ligated into the EcoRI-BglII-digested pHWO18 vector backbone to form pGC199. All these expression vectors were linearized at the StuI site in *P. pastoris HIS4* and transformed into JC132 and GS115. Purified colonies were induced and assayed for β-lactamase, alcohol oxidase, and glyceraldehyde-3-phosphatase activities as described above.

Plasmid constructions. For complementation studies, a 4.4-kb fragment containing the entire *MXR1* gene was amplified from pYL1 using the primers MXRPR1 and GPC3TT. The fragment was digested with BamHI and KpnI and inserted into the same sites in pBLHIS (5) to create pGC213 using SURE *E. coli* cells (Stratagene, La Jolla, CA). pGC213, containing *MXR1* and *HIS4*, was transformed into JC132, and the resulting Mut$^+$ colonies were isolated as the complemented *mxr1* strains in further studies. pGC215 was created by amplifying the 3.5-kb coding sequence of *MXR1* with the primers MXRATG and MXRTAG. The PCR product was digested with BamHI and XbaI and ligated into the BamHI and AvrII sites of pPIC3K to create pGC215, in which *MXR1* transcription is regulated by the *AOX1* promoter. pGC216 was created by filling in the ends of the 3.5-kb PCR fragment of pGC215 with a Klenow fragment, digesting it with XbaI, and inserting it into the PmlI and XbaI sites of pJS1 (a gift of Jay Sunga, Keck Graduate Institute, Claremont, CA). The resulting construct contained the *MXR1* gene under the regulation of the *PEX8* promoter with the zeocin resistance gene as a selectable marker. To create pGC217, the stop codon of *MXR1* was mutagenized in pGC216 to provide an in-frame fusion with the c-*myc* epitope and His_6 tag.

Northern blotting. Yeast RNAs were prepared by a standard procedure adapted for total yeast RNA isolation (4). Total RNA concentrations of each sample were determined spectrophotometrically (4). Samples containing 10 μg of total RNA were loaded into the wells of 1.5% agarose denaturing formaldehyde gels and electrophoresed for separation. Transfer of RNA to MagnaGraph nylon membranes (Osmonics, Westborough, MA), cross-linking, prehybridization, hybridization, high-stringency washing, and imaging were all performed according to a standard procedure (41). The nylon membranes were washed and visualized according to the instructions for the Southern-Light detection kit (Tropix, Bedford, MA).

TABLE 2. Oligonucleotide primers used for PCR in this study

Primer	Sequence
MXRATG	5′-CACGGATCCACGCACAATGAGCAATCTAC-3′[a]
MXRPR1	5′-CAAGGATCCGGTGTCATCAAGTGGCGTACTG-3′[a]
MXRP2	5′-CGAGGATCCTGATCGGAACCCTGACC-3′[a]
MXRR1	5′-CCGGAATTCTGTGCGTGGGATAAAGTCATC-3′[b]
MXR1MUT	5′-CTAGATGGTGGTGTCTACCTAGAACAAAAACTC ATCTC-3′
MXRTAG	5′-CCAATCTAGACTAGACACCACCATCTAGTC-3′[c]
GPC3TT	5′-TAGGTACCGAATGGGTCAGCAGAATAC-3′
HIS4C1	5′-AAATACGCTGATCCAGATTTCG-3′
AOXPB1	5′-ACGGAGATCTACCGTTTGTCTTGTTTGG-3′[d]
AOXPB1.2	5′-GGCAGATCTAAAAATAATCTCATTAATGC-3′[d]
AOXPB1.3	5′-GGCAGATCTAATCTCATTAATGCTTAGCG-3′[d]
AOXPB1.4	5′-ACGGAGATCTCGCTTCTGAACCCGGTGGC-3′[d]
AOXPB2	5′-CGGAGATCTAACAACCCGCTTTTTGGATG-3′[d]
AOXPB2.1	5′-GGCAGATCTGATTATGCATTGTCCTCCAC-3′[d]
AOXPB2.3	5′-GGCAGATCTATTGTATGCTTCCAAGATTC-3′[d]
AOXPB2.5	5′-ACGGAGATCTGGTGGGAATACTGCTG-3′[d]
AOXPB3	5′-ACGGAGATCTGTTCTAACCCCTACTTG-3′[d]
AOXPE	5′-CCGGAATTCCTCGTTTCGAATAATTAG-3′[b]
SUFFSO	5′-CGGACTAGTGAAACCTTTTTGCCATCC-3′[e]
SUFFS1	5′-CGGACTAGTTACCGTTTGTCTTGTTTGGT-3′[e]
SUFF1.4X	5′-CCGCTCGAGAAGCGATAGAGAGACTGC-3′[f]
SUFFS1.5	5′-CGGACTAGTTCTGAACCCGGTGG-3′[e]
SUFF2.5X	5′-CCGCTCGAGTCTTGGAAGCATACAATG-3′[f]
SUFF2.7X	5′-CGGCTCGAGTTTTGATCATGAACGT-3′[f]
SUFFS3	5′-CGGACTAGTTCTGGTGGGAATACTGCTG-3′[e]
SUFFX	5′-CCGCTCGAGGAAGTAGGGGTTAGAACAG-3′[f]
LEU1X	5′-CGGCTCGAGTATTATTTAAGGACCTATTG-3′[f]
LEUR5	5′-TTCGGATAATGCGAACAG-3′
LACZX3	5′-CCGGAATTCACCGAACATCCAAAAGTTTGTG-3′[b]
LACZ5	5′-ATCATCCTCTGCATGGTCAGG-3′
GAP1A	5′-TTATTTGTCCCTATTTCAATC-3′
GAP2	5′-AGCTATTTCACATACAAATCG-3′
DAS5A	5′-TCACGGTTCTGCTCTTGGTG-3′
DAS3A	5′-CAACTACTAACCCGTTAGTG-3′
PFLD-7	5′-GCTTGTTCATACAATTCTTG-3′
FLD2	5′-CGGAATTCTTAGTGCATAGTAATCACAG-3′
KR323	5′-ATATTTGCAGCGATGATC-3′
KR326	5′-AATTTCCCTTTTGAGGGTCAT-3′
THIO5	5′-GATGGAAAGACTTTCACAAC-3′
THIO3	5′-CTATTCTCTAGCAAACACTGC-3′
OMTF5.1	5′-GAGAACAACCTCAACAACC-3′
OMTR5.1	5′-CATCGTAATCAGAAGCAGAAC-3′

[a] The BamHI site is underlined.
[b] The EcoRI site is underlined.
[c] The XbaI site is underlined.
[d] The BglII site is underlined.
[e] The SpeI site is underlined.
[f] The XhoI site is underlined.

Biotinylated DNA probes were synthesized utilizing the BioPrime DNA labeling system (Invitrogen, Carlsbad, CA). Template DNAs were generated by PCR using the primers indicated in Table 2 or by gel isolation after restriction digestion with the Geneclean II kit (Qbiogene, Carlsbad, CA). Labeled DNAs were separated from unincorporated label by using the QIAquick PCR purification kit (QIAGEN, Valencia, CA). Approximately 400 ng of purified biotinylated DNA fragments was denatured by heating at 95°C for 5 min, chilled on ice for 2 min, and then added as hybridization probes to 10-cm by 10-cm blots.

The probe for *AOX1* was a 1.4-kb BamHI-StuI fragment from pPG4. The *PEX5* probe (49) was created from the template of a 1.8-kb BglII-EcoRI fragment of pSP72-PAS8. The *PEX8* probe was created using the 740-bp PCR product amplified from pK316 with the primers KR23 and KR326. Both pSP72-PAS8 and pK316 were generously provided by Kim Russell (Oregon Graduate Institute, Portland, OR). The *PEX14* probe was a 1.1-kb EcoRI-HindIII fragment from pMJ14 (25). The 1.1-kb probe for *P. pastoris GAP* was generated by using the primers GAP1A and GAP2 on pGAP9 (58). The *MXR1* probe was created by amplifying the 3.5-kb coding sequence in pYL1 with the primers MXRATG and MXRTAG. The *DHAS* probe was synthesized by amplifying a 1.3-kb fragment from pYS3 (43) with primers DAS5A and DAS3A. The *FLD1* probe was generated by amplifying a 1.2-kb fragment from pYG1 (43) with the primers PPFLD-7 and FLD2. The 1.3-kb thiolase probe was produced using the primers THIO5 and THIO3 to amplify a region of its coding sequence from pBlu-thiolase (provided by S.S.).

Electron microscopy. Electron microscopy was performed as described previously (59).

Fluorescence microscopy. Cells of *P. pastoris* strain SMY175 (*MXR1-HA kan*::*MXR1 his4 arg4*) were precultured in YPD medium and, in mid-logarithmic phase, shifted to YND, YNM, or YNO medium supplemented with 20 μg/ml histidine and arginine. Defined medium cultures were inoculated at 0.2 OD_{600} units/ml (YNG) or 0.8 OD_{600} units/ml (YNM and YNO). Cells were harvested for microscopy after overnight growth. Samples were prepared as described previously (36), with some modifications. Briefly, cells were fixed with 4% paraformaldehyde, spheroplasted with Zymolyase 20T, and then adhered onto a polylysine-coated glass slide followed by acetone postfixation at −20°C. Samples were rehydrated in phosphate-buffered saline (PBS) block (4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.4, with 1% skim milk, 0.1% bovine serum albumin, and 0.1% *n*-octyl glucoside) for 30 min and then incubated with primary anti-hemagglutinin (HA) antibody (Covance) (1:2, 000 dilution in PBS block). After incubation overnight at 4°C, samples were washed with PBS block and incubated with secondary Alexa Fluor-labeled anti-mouse immunoglobulin G antibody (Molecular Probes) (diluted 1:200 in PBS block). After a 1-h incubation in the dark, samples were washed with PBS block. A drop of mounting medium (95% glycerol, 0.1% *p*-phenylenediamine, 2.5 μg/ml DAPI [4′,6′-diamidino-2-phenylindole]) was added, and then a coverslip was placed over the sample. Samples were viewed with an Axioskop 2 fluorescence microscope equipped with rhodamine and DAPI filters (Zeiss).

Deletion and sufficiency analysis of the *AOX1* promoter. The *AOX1* promoter deletion series, utilized for identifying necessary regions of the promoter, was constructed as follows. pHW018 (58) contains 735 bp of the *AOX1* promoter region upstream of the translation initiation codon of a modified β-lactamase coding sequence. The 735-bp region is contained between BglII and EcoRI. This promoter region was excised by digestion with these enzymes and replaced with PCR products harboring progressive 5′-to-3′ deletions of this upstream region. For consistency, pHW018 was renamed pHWG0. All plasmids with the pHWG label contain deletions of the 735-bp 5′ upstream region, which were sequenced to confirm their authenticity.

To construct plasmids to test sufficiency, the 3.3-kb fragment of pLG178F (a gift of Leonard Guarente, Massachusetts Institute of Technology, Cambridge, MA) containing the *Saccharomyces cerevisiae CYC1* TATA box fused to a partial *lacZ* coding sequence was inserted into the same restriction sites in pBLHIS (5) to create pGC140. The 350-bp EcoRI-HindIII fragment containing the *AOX1* transcription termination sequence of pHILD2 (Invitrogen, Carlsbad, CA) was then inserted into the same sites of pGC140 to produce pGC141. A 1.3-kb PCR fragment containing the *S. cerevisiae LEU2* TATA box fused to the 5′ end of the *lacZ* coding sequence was amplified using primers LEU1X and LEUR5, digested with XhoI and EcoRV, and used to replace the XhoI-EcoRV fragment of pGC141 to construct pGC146. The full-length *lacZ* coding sequence was cloned into pGC146 by amplifying a 2.1-kb fragment from pMC2019 (a gift of Malcolm Casadaban, University of Chicago, Chicago, IL) and inserting it into the EcoRV and EcoRI sites of pGC146 to produce pGC187. pGC187 contains the basal promoter of *S. cerevisiae LEU2*, with no upstream promoter region, fused to the complete coding sequence of *lacZ*, followed by the *AOX1* transcription termination region. pGC182 was produced by inserting the 243-bp *AOX1* promoter region created by PCR with the primers SUFFS1 and SUFFX into the SpeI and XhoI sites of pGC187.

All plasmids constructed for deletion and sufficiency analysis were linearized in the *P. pastoris HIS4* gene and transformed into GS115 and JC132. Colony PCR was employed to confirm the presence of the expression vectors in the transformed strains (5). Strains were assayed for β-lactamase or β-galactosidase activity on glucose- or methanol-containing medium as described above.

Mxr1p overexpression. JC132 and GS115 were transformed with both pJS1 and pGC217 (see plasmid constructions described above). High- and low-copy-number strains were isolated based on zeocin resistance (*Pichia* EasySelect Expression kit; Invitrogen, Carlsbad, CA) and verified by Southern analysis. Strains were tested for the ability to grow on medium containing methanol as a sole carbon source. Strains were grown to an OD_{600} of approximately 0.5 on YND medium. Cells were then centrifuged and suspended in YNM medium to induce *PEX8* promoter expression. Cell extracts were made from YND cultures as well as YNM cultures at 12-h increments. Western analysis with the anti-*myc* antibody was performed to determine the level of Mxr1p-His_6-*myc* in the cellular extracts.

Band shift assays. Cell extracts were made from GS115 containing the pGC217 plasmid (Mxr1p overexpression plasmid) and JC133 (*mxr1*Δ::*HIS4*) strains as described previously (37). Briefly, cells were grown on YPD medium to an OD_{600} of ~1.0, centrifuged, washed with sterile water, and then suspended in an equivalent volume of YNM medium. Approximately 50 ml of cells was induced on methanol medium for 6 h and harvested by centrifugation. Pellets were resuspended in 400 μl breaking buffer (0.2 M Tris-HCl, 10% glycerol, 10 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM EDTA, 0.2 μg/ml leupeptin, 0.2 μg/ml pepstatin) with ~400 μl acid-washed 0.45- to 0.60-mm glass beads. Cells were vortexed for 30 min at 4°C and subsequently centrifuged for 30 min at 4°C. The cell extract supernatant was transferred to fresh 1.5-ml tubes and stored at −80°C. The 150-bp and 243-bp *AOX1* fragments were amplified from pHWG0 using the primers OMTF5.1/OMTR5.1 and SUFFS1/SUFFX, respectively. The PCR mix was supplemented with 2.0 mM deoxynucleoside triphosphates plus 0.5 mM biotin-labeled dUTP (Molecular Probes, Eugene, OR). A total of 1.0 ng biotin-labeled fragments was mixed with 10 μg extract protein, breaking buffer, and 10% glycerol, with or without 100 ng poly(dI-dC) and with or without 100 ng unlabeled *AOX1* fragments; 10% nondenaturing Tris-glycine PAGE gels were prerun for ~2 h at 100 V and subsequently loaded with samples and run for ~2 h at 100 V. Gels were transferred to MagnaGraph nylon membranes with a Miniprotean II blotter for 45 min at 80 V according to the manufacturer's instructions. Membranes were washed with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), dried for 5 min at 70°C, and subsequently UV cross-linked with 1,200 mJ of radiation. Membranes were probed using the BrightStar BioDetect kit (Ambion, Austin, TX) and then visualized by autoradiography.

Miscellaneous methods. Recombinant DNA methods were performed essentially as described previously by Sambrook et al. (41). Plasmid DNA digested with restriction enzymes and used for restriction mapping, hybridization probes, and cloning of subfragments was separated on Tris-borate-EDTA agarose gels. DNA fragments were purified from agarose gels by using a Schleicher and Schuell (Keene, NH) NA45 DEAE membrane or the Geneclean II kit (Qbiogene, Carlsbad, CA). Restriction enzymes were purchased from New England Biolabs, Inc. (Beverly, MA). Site-directed mutagenesis was executed by using the Transformer Mutagenesis kit (Clontech, Palo Alto, CA). All mutated sites were confirmed by sequencing. Oligonucleotides were synthesized by Sigma Genosys (Plano, TX), and DNA sequencing was performed at the Oregon Regional Primate Research Center, Molecular Biology Core Facility (Beaverton, OR). Protein alignments were performed with ClustalW. The alignment of *MXR1* and *S. cerevisiae ADR1* sequences and the search for potential biologically significant sequences in both proteins were done using PC/Gene software (release 6.8; Intelligenetics, Mountain View, CA).

RESULTS

Isolation of the *P. pastoris mxr1-1* mutant and cloning of the *MXR1* gene. Mutants in *mxr1* were initially isolated as part of a search for strains defective in peroxisome biogenesis (*pex* mutants) (26). Like *pex* mutants, *mxr1* mutants were defective in growth on methanol and oleic acid but were able to grow on other carbon sources including glucose, glycerol, and ethanol (Table 3). However, unlike *pex* mutants, *mxr1* mutants could not induce transcription of *AOX1* or an 5′ *AOX1-lacZ* reporter in response to methanol (see, for example, Fig. 2B), a result that suggested that these mutants were not affected in peroxisome biogenesis but in a positive regulatory factor required for a response to methanol (and possibly also oleate). Based on these initial studies, we named the affected gene *MXR1* (for methanol expression regulator 1).

To identify the affected gene, an *mxr1-1 his4* strain (JC132) was transformed with a *P. pastoris* genomic library, and numerous His^+ Mut^+ colonies were obtained. However, attempts to recover the transforming plasmids from complemented yeasts by transformation of their total genomic DNAs into bacteria were unsuccessful. As an alternative plasmid identification and recovery strategy, we employed a Sib selection method (3, 31) (see Materials and Methods for details), which was successful. The complementing and recovered plasmid, named pYL1, contained a *P. pastoris* genomic DNA insert of approximately 14 kb. Subcloned portions of this insert identified a DNA

TABLE 3. Generation times of *mxr1* mutants on selected carbon sources

Strain	Generation time (h:min)					
	Glucose (rich, YPD)	Glucose (minimal, YND)	Glycerol (minimal, YNG)	Ethanol (minimal, YNE)	Methanol (minimal, YNM)	Oleate (minimal, YNO)
WT[a]	1:50	2:10	2:20	3:30	4:30	6:15
mxr1-1	2:35	3:45	4:30	6:00	No growth	No growth
*mxr1*Δ	2:30	3:45	3:45	6:00	No growth	No growth
mxr1-1/*MXR1*	2:00	2:15	2:25	3:30	4:30	6:30

[a] WT, wild type.

fragment of approximately 7 kb that complemented the *mxr1-1* strain, which was subsequently sequenced.

The predicted amino acid sequence of Mxr1p shows similarities to that of Adr1p. DNA sequencing results revealed one large (3,468 bp) open reading frame (ORF) with the potential to encode a protein of 1,156 amino acids, and further studies, described below, with a constructed null strain demonstrated that this ORF was *MXR1*. Strikingly, a ~70-amino-acid segment (amino acids 29 to 101) near the predicted N terminus of Mxr1p showed strong similarity (70% identity and 82% similarity) to a region at a similar position within *Saccharomyces cerevisiae* Adr1p (alcohol dehydrogenase II synthesis regulator), a transcription factor necessary for growth of baker's yeast on ethanol, glycerol, and oleate (Fig. 1) (15, 62). Both sequences also contained two type I C_2H_2 DNA-binding zinc fingers in this area. Outside this ~70-amino-acid region of similarity, additional but more-modest sequence similarities between Adr1p and Mxr1p were observed, such as potential phosphorylation sites for cyclic AMP (cAMP)-dependent protein kinase and protein kinase C (Fig. 1) (7, 8, 52). Interestingly, one similarity was a 58-amino-acid region (residues 406 to 463 in Adr1p) (14% identity and 40% similarity) that included the mini-Adr1p domain, a 43-residue domain (residues 420 to 462) that has been shown to be able to execute all the transactivation functions of wild-type Adr1p (62). Another alignment program found 36% identity and 57% similarity between a different Mxr1p region and this 58-amino-acid Adr1p segment. These sequence observations suggested that Mxr1p may be a transcription factor required for the activation of genes involved in methanol and oleate utilization.

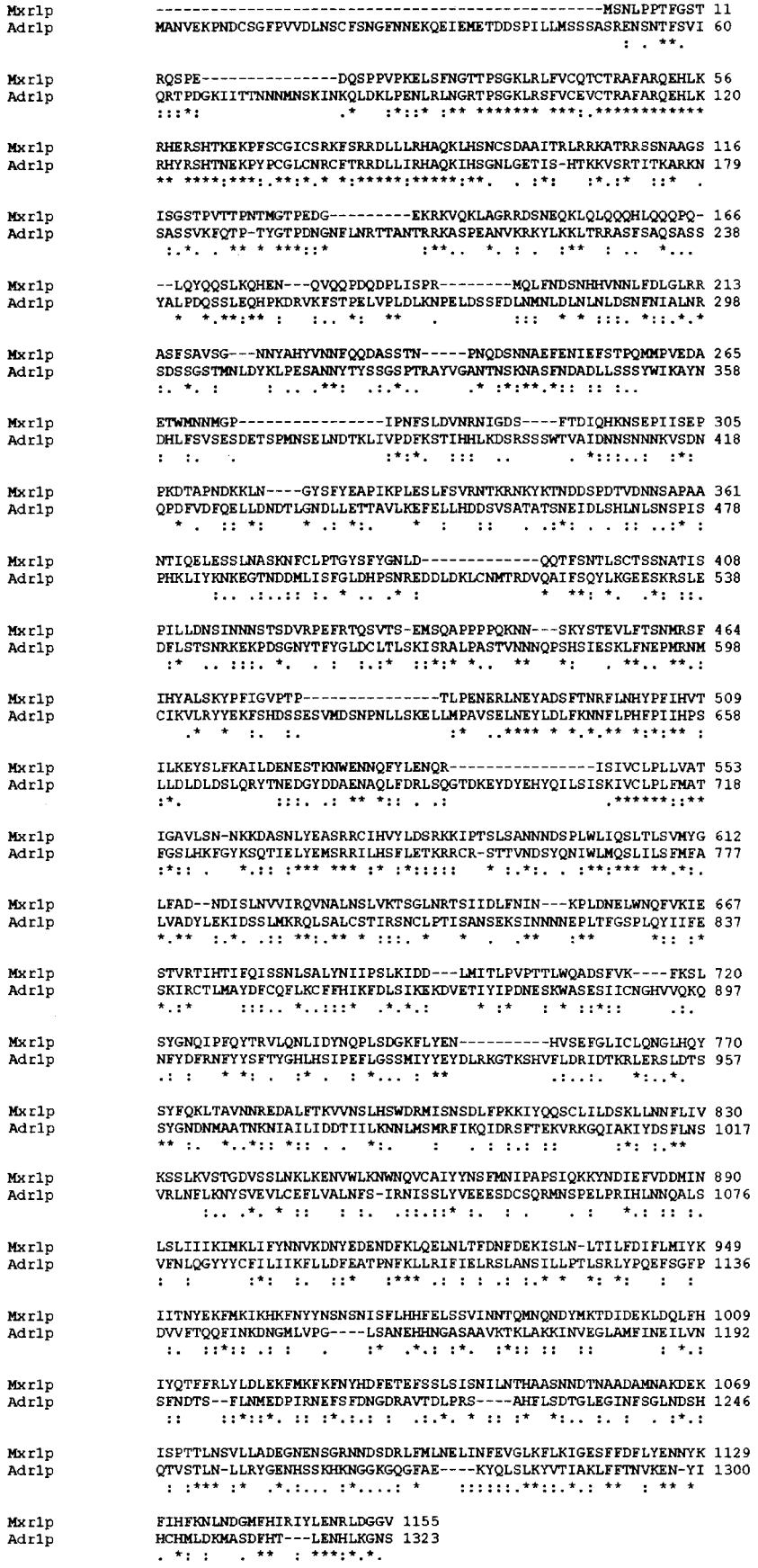
To confirm that the ORF identified above was *MXR1* and to examine the phenotype of a null allele of *mxr1*, we constructed a *P. pastoris* strain in which the first 1,002 amino acids of the *MXR1* ORF were deleted. This *mxr1*Δ strain was prepared by first constructing a plasmid, pGC202, in which this region of the *MXR1* ORF had been removed and replaced with a DNA fragment encoding the *P. pastoris HIS4* gene. A fragment from this plasmid was then transformed into *P. pastoris* strain GS115 (*his4*) by selection for histidine prototrophy (His^+), and His^+ transformants were screened for ones that were also methanol utilization defective (Mut^-). Deletion of the putative *MXR1* ORF in the resulting strains was confirmed by colony PCR (data not shown). One *mxr1*Δ::*HIS4* strain (*mxr1*Δ; JC133) was selected for further studies. RNA extracted from the *mxr1*Δ strain and hybridized with a DNA fragment containing sequences from *MXR1* demonstrated that this strain was devoid of *MXR1* message (Fig. 2B). The *mxr1*Δ strain was also unable to grow on oleate, confirming that both methanol and oleate growth-deficient phenotypes were the result of mutations in one gene (Table 3). A derivative of the *mxr1*Δ strain, JC134, was crossed with the original chemically induced strain, JC132 (*mxr1-1*), and all resulting diploid lines and their haploid spore products were observed to be Mut^-. These results demonstrated that the mutation in our *mxr1-1* and *mxr1*Δ strains were tightly linked and most probably mutant alleles in the same gene and therefore that the cloned ORF was most likely *MXR1*.

Mutants in *mxr1* are defective in growth on the peroxisomal substrates methanol and oleate. As a first step in elucidating the function of *MXR1*, we examined the phenotype of our *mxr1* mutants. For these studies, four haploid strains were examined in parallel: JC100 (wild type); JC132 (*mxr1-1*), JC133 (*mxr1*Δ), and JC135 (*mxr1-1* complemented with a plasmid, pGC213, containing *MXR1*). We first examined the ability of these strains to utilize other carbon sources (Table 3). In addition to their complete inability to grow on methanol and oleate, the *mxr1* strains showed retarded growth rates, relative to the wild-type or complemented strains, on all carbon sources tested, especially glycerol and ethanol, where their generation times were nearly twice the normal generation times. The longer doubling times on all carbon sources examined suggested that Mxr1p also plays a role (albeit a minor one) in the expression of one or more genes other than those required for growth on methanol and oleate.

Mxr1p is required for expression of methanol pathway proteins and genes. We next examined cell extracts prepared from methanol-induced cultures of our *mxr1* strains for activity levels of selected methanol pathway enzymes. As seen in Table 4, the lack of Mxr1p function resulted in a significant decrease in specific activity for several methanol utilization enzymes, with Aox showing the most extreme effect (>1,000-fold) and formaldehyde dehydrogenase and catalase displaying the least extreme effect (<5-fold). As a control, activity levels for the glycolytic enzyme glyceraldehyde-3′-phosphate dehydrogenase (Gap) were nearly unaffected in the mutants.

Western blot studies were performed on protein extracts prepared from glucose-grown and methanol-induced cultures of the same strains. Samples of each extract were separated by SDS-PAGE and reacted with antibodies against selected methanol pathway enzymes and peroxins. These experiments showed that a loss of *MXR1* function significantly reduced levels of Aox and Cat proteins but only modestly decreased levels of the peroxins Pex5p and Pex14p (Fig. 2A).

Evidence that these reductions in selected protein levels were due to reduced transcription of their genes was obtained in Northern blot studies performed on RNAs extracted from glucose-grown and methanol-induced cultures of the *mxr1* mu-



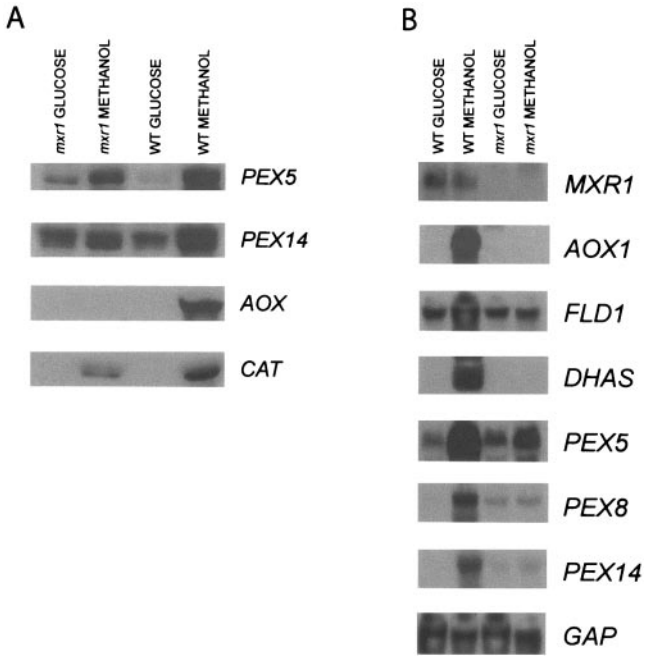


FIG. 2. *MXR1* is necessary for normal accumulation of peroxin and methanol utilization proteins and messages under methanol induction conditions. (A) *mxr1-1* and wild-type (WT) cells were grown to an OD_{600} of 0.5 on glucose, shifted to methanol-based media for 8 h, and harvested. Cell extracts were prepared and analyzed by Western analysis using the Tropix Western-Light detection kit. *PEX5*, peroxin 5; *PEX14*, peroxin 14; *AOX1*, alcohol oxidase I; *CAT*; catalase. (B) Wild-type and Δ*mxr1* strains were grown on glucose to an OD_{600} of 0.5, maintained on glucose or shifted to methanol for 8 h, and harvested. Total RNA was extracted, run on formaldehyde-agarose gels, and blotted onto a nylon membrane. The Northern blots were hybridized with biotin-labeled probes and subsequently processed with the Tropix Southern-Star detection system. *MXR1*, methanol expression regulator 1; *DHAS*, dihydroxyacetone synthase; *FLD1*, formaldehyde dehydrogenase; *PEX8*, peroxin 8; *GAP*, glyceraldehyde-3-phosphate dehydrogenase.

tants. Steady-state message levels from the methanol pathway genes *AOX1*, *DHAS*, and *FLD1*; the peroxin genes *PEX5*, *PEX8*, and *PEX14*; and a control gene, *GAP*, were measured. As shown in Fig. 2B, *MXR1* function did not appear to be necessary for setting the basal level of expression of these mRNAs in glucose medium, since comparable levels of each mRNA species were seen in both wild-type and mutant cells grown in glucose. However, *MXR1* function was essential to varying degrees for increased steady-state levels of these messages in methanol medium. At one extreme, *MXR1* function appeared to be essential for the large accumulations of the *AOX1* and *DHAS* transcripts, while accumulation of *PEX5* and *FLD1* messages appeared to be much less dependent.

To examine whether the reduced mRNA levels were most likely due to a reduced rate of transcription initiation in the absence of *MXR1* (as opposed to an increased message degradation rate), we fused the promoter regions from the *AOX1*, *PEX8*, and *GAP* genes to the protein-coding sequences of a sensitive transcriptional reporter derived from the bacterial β-lactamase (*bla*) gene (58). Each promoter-*bla* fusion (P_{AOX1}-*bla*, P_{GAP}-*bla*, and P_{PEX8}-*bla*) was introduced into both wild-type and *mxr1* mutant strains, and glucose-grown and methanol-induced cells were assayed as described above (Fig. 3). In wild-type cells, reporter fusion data indicated that both P_{AOX1} and P_{PEX8} promoters were strongly activated by a shift to methanol medium and to levels similar to those observed in our Northern data (~1,000× and 10×, respectively). In contrast, no significant activation of these promoters was evident in the *mxr1*mutant shifted to methanol medium. The basal level of reporter expression in glucose was unchanged in *mxr1* cells compared to *MXR1* cells for both of these promoters. This regulation mirrors what we observed in our Northern blots (Fig. 2B). In contrast, the levels of expression of the control reporter P_{GAP}-*bla* were similar in both wild-type and *mxr1* strains. These results indicate that *MXR1* has little effect on the expression of housekeeping genes such as *GAP*, implying that *MXR1* function is primarily involved with a subset of genes, specifically those related to methanol utilization and peroxisome biogenesis in response to methanol.

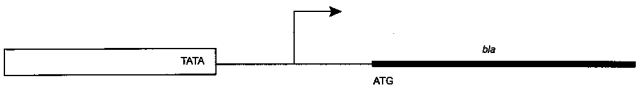
Peroxisomal structures in the *mxr1-1* mutant under methanol-inducing conditions were examined by electron microscopy. In methanol-induced cells of *mxr1-1*, the large, numerous peroxisomes seen in wild-type *P. pastoris* appear absent (see Fig. 5B and D). Taken together with the Western and Northern data, these results suggest that Mxr1p function is essential for the expression of a subset of genes related to methanol utilization and peroxisomal biogenesis in response to methanol.

Mxr1p function is also required for oleate utilization. Because *mxr1* mutants also cannot grow on oleic acid, the effect of the loss of *MXR1* function was examined on selected β-oxidation pathway enzymes (acyl-CoA oxidase [Aco] and catalase [Cat]). To examine this, strains were grown on glucose medium, shifted to oleate medium, and harvested. In contrast to the methanol pathway enzymes, specific activities of Aco and Cat were only modestly decreased in the *mxr1* mutant strains (Table 5). Similar results were seen in Western blots for Pex5p and Pex8p; i.e., levels of these peroxins were only slightly lower

TABLE 4. Specific activities of methanol pathway enzymes in *mxr1* mutant cells

Strain	Sp act of enzyme (% of wild type)				
	Aox	Cat	Fdh	Fld	Gap
Wild type	100	100	100	100	100
*mxr1*Δ	<0.05	23.4	6.3	35.6	102
mxr1-1	<0.05	31.7	7.9	22.4	79.2
mxr1-1/MXR1	55.7	104	73.6	121	83.1

FIG. 1. Computer comparison of the predicted *P. pastoris* Mxr1 and *S. cerevisiae* Adr1 proteins indicates a 70% amino acid identity and an 82% similarity within 73 amino acids of the N termini of the two peptides. ClustalW alignment was performed on the predicted amino acid sequence of *MXR1* and the amino acid sequence of *ADR1*. Stars represent identical matches, while dots represent conservative changes. The region of similarity is predicted to encode two C_2H_2-type zinc finger domains.



b-LACTAMASE ACTIVITIES

REPORTER	WT GLUCOSE	WT METHANOL	*mxr1* GLUCOSE	*mxr1* METHANOL
$P_{AOX1\text{-}bla}$	<0.5	300	<0.5	<0.5
$P_{PEX8\text{-}bla}$	7.9	76.4	3.6	2.5
$P_{GAP\text{-}bla}$	398	214	196	113

FIG. 3. Promoters of methanol-responsive genes require *MXR1* function for transcriptional induction. Wild-type (WT) and *mxr1* strains bearing the illustrated promoter-reporter constructs were grown on glucose to an OD_{600} of 0.5, maintained on glucose (G) or shifted to methanol (M) for 8 h, and harvested. Cell extracts were generated by glass bead lysis and assayed for β-lactamase activity and protein concentration to produce specific activity values. β-Lactamase specific activity is expressed as nanomoles per milligram per minute.

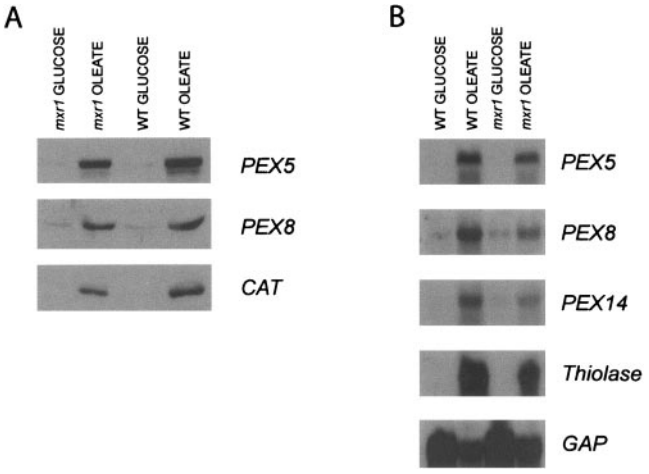


FIG. 4. *mxr1* cells show normal accumulations of peroxin and β-oxidation proteins and messages under oleate induction conditions. (A) *mxr1-1* and wild-type (WT) cells were grown to and OD_{600} of 0.5 on glucose, shifted to oleate-based medium for 8 h, and harvested. Cell extracts were prepared and analyzed by Western analysis using the Tropix Western-Light detection kit. (B) Wild-type and Δ*mxr1* strains were grown on glucose to an OD_{600} of 0.5, maintained on glucose or shifted to oleate for 8 h, and harvested. Total RNA was extracted, run on formaldehyde-agarose gels, and blotted onto a nylon membrane. The Northern blots were hybridized with biotin-labeled probes and subsequently processed with the Tropix Southern-Star detection system.

in *mxr1* oleate-induced cells relative to those of the wild type (Fig. 4A).

We extended this analysis to mRNA levels. In addition to *PEX5* and *PEX8*, we also examined message levels for a third β-oxidation pathway enzyme, thiolase, and for the peroxin gene *PEX14* (Fig. 4B). Similar to the protein data described above, mRNA levels for these genes were only slightly reduced in oleate-induced *mxr1* cells relative to those of the wild type.

Since β-oxidation enzyme and peroxin levels appeared normal in oleate-induced *mxr1* cells, we considered the possibility that the mutant cells were unable to grow in oleate due to some other malfunction of the peroxisomal import machinery resulting in the mislocalization of one or more β-oxidation enzymes, all of which are normally peroxisomal. To examine this, oleate-induced cells of the *mxr1*Δ strain were harvested, homogenized, and fractionated into a cytosolic supernatant and crude organelle pellet fraction containing mainly mitochondria and peroxisomes. Activities for Cat and Aco were then measured in the resulting fractions. Cytochrome *c* oxidase, a mitochondrial marker protein, was used as a control to confirm the general integrity of the organelles in the pellet. As shown in Table 6, Aco and Cat activities were located primarily in the peroxisomal pellet, as in the wild type, indicating normal localization for these enzymes in the *mxr1* cells. Finally, thin sections of cells from an oleate-induced *mxr1* strain were examined by electron microscopy, and peroxisomes were observed to be similar to those of the wild type with regard to size, number, and protein content (Fig. 5A and C). Thus, although *mxr1* cells cannot grow on oleate, we were unable to establish a cause at the molecular level for this deficiency.

***MXR1* is expressed constitutively at a low level.** We examined the regulation of *MXR1* expression via Northern blot hybridization of a labeled *MXR1* fragment to RNA extracted from glucose- and methanol-grown *P. pastoris* cells. As shown in Fig. 2B, these Northern blots showed low levels of *MXR1* message in both glucose- and methanol-grown cells, indicating that *MXR1* is constitutively expressed at a minimal rate in *P. pastoris*. We also conducted reporter gene studies using a *P. pastoris* strain that contained a vector with an approximately 700-bp region from the 5′ end of the *MXR1* gene (P_{MXR1}) fused to *bla* encoding β-lactamase (P_{MXR1}-*bla*). The reporter results were consistent with the Northern blots and showed a less-than-threefold increase in *MXR1*-controlled reporter gene activity in response to methanol in wild-type cells (Table 7). For comparison, in methanol-grown wild-type cells, a P_{AOX1}-*bla* reporter generated β-lactamase specific activity levels ap-

TABLE 5. Specific activities of β-oxidation enzymes in oleate-induced cells

Strain	Sp act of enzyme (% of wild type)			
	Aox	Cat	Acyl-CoA oxidase	Gap
Wild type	100	100	100	100
*mxr1*Δ	<1.0	37.6	43.5	102
mxr1-1	<1.0	36.9	24.8	79.2
mxr1-1/*MXR1*	28.9	97.9	111	83.1

TABLE 6. Specific activity of β-oxidation pathway enzymes in crude organelle fractions

Strain	Sp act of enzyme[a]			
	Fraction	Cat	Aco	Cyt *c* oxidase
Wild type	Pellet	15.9	0.812	0.443
	Supernatant	12.7	0.0612	0.0198
*mxr1*Δ	Pellet	5.16	0.598	0.601
	Supernatant	3.61	0.024	0.0586

[a] Catalase activity units are expressed as ΔE_{240}/minute/milligram; acyl-CoA oxidase units are expressed as micromoles of product/minute/milligram; cytochrome *c* (Cyt *c*) oxidase units are expressed as micromoles of product/minute/milligram.

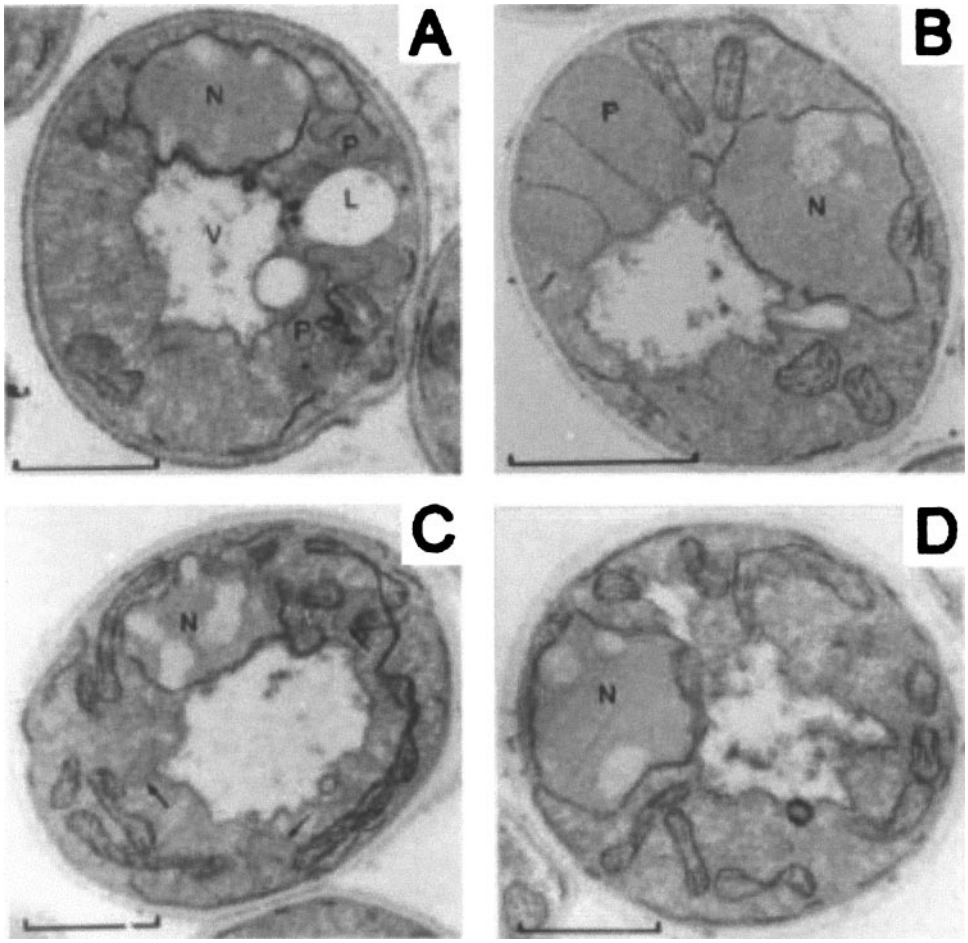


FIG. 5. Peroxisome proliferation is found in *mxr1* cells grown under oleate-inducing conditions but not under methanol-inducing conditions. Electron microscopy was used to visualize the intracellular organelles of (A) wild-type cells grown on oleate medium, (B) wild-type cells grown on methanol medium, (C) *mxr1* cells grown on oleate medium, and (D) *mxr1* cells grown on methanol medium. P, peroxisome; N, nucleus; V, vacuole; L, lipid body.

proximately 1,000-times higher than those seen in the P_{MXR1}-*bla* strain (Fig. 3).

Identification of the transcriptional regulatory regions in the *AOX1* promoter. Because of the similarity of the Mxr1p sequence to known DNA-binding transcription factors such as Adr1p and the effect of its absence (in *mxr1* mutants) on the expression of genes involved in methanol utilization, we hypothesized that Mxr1p may be a transcription factor that helps activate certain methanol pathway and peroxin genes in *P. pastoris*. To examine this hypothesis directly, we performed DNA band shift studies in which a DNA fragment from the *AOX1* promoter, a gene whose transcription is highly dependent on Mxr1p, was incubated with extracts from *P. pastoris* strains containing Mxr1p.

TABLE 7. Specific activity of P_{MXR1}-*bla* reporter

Strain	Sp act (nmol/mg/min)	
	Glucose	Methanol
Wild type	0.07	0.20
mxr1-1	0.06	0.30

As a preliminary step in these studies, we first needed to identify the approximate location of sequences upstream of *AOX1* that are critical to its promoter function and therefore the probable site of Mxr1p binding. To accomplish this task, we began by performing a 5′-to-3′ deletion analysis of the *AOX1* upstream region by constructing a family of vectors derived from pHWG0 (Fig. 6A) that were identical except that each contained an *AOX1* upstream fragment with a different 5′ deletion end point. Deletion end points were selected based on homology between *AOX1* sequences and defined *cis*-acting sequences in yeast genes that are modulated by a carbon source (29, 35). Each *AOX1* upstream fragment was inserted into the vector and positioned in front of the bacterial *bla* gene so that the transcriptional activity could be evaluated by measuring the level of β-lactamase activity present. The largest *AOX1* upstream fragment, pHWG0, terminated at its 5′ end with a natural SacI site and included 735 bp 5′ of the translational start site of *AOX1* (−735). This fragment is the same one that is utilized in *P. pastoris* heterologous expression vectors and appears to contain all critical *cis*-acting sequences needed for P_{AOX1} function (53). The 3′ ends of all *AOX1* upstream fragments were identical and included the putative *AOX1*

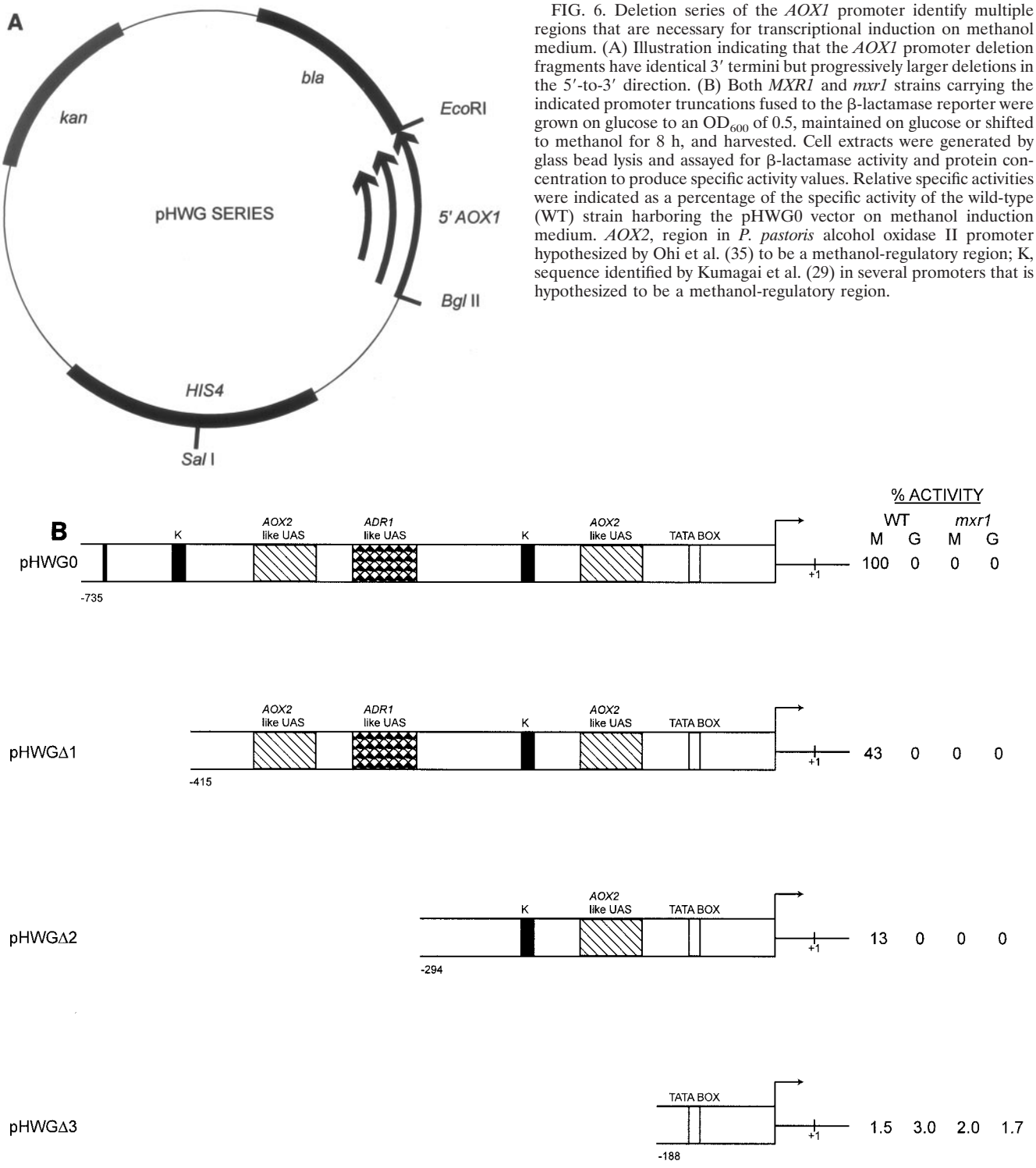


FIG. 6. Deletion series of the *AOX1* promoter identify multiple regions that are necessary for transcriptional induction on methanol medium. (A) Illustration indicating that the *AOX1* promoter deletion fragments have identical 3′ termini but progressively larger deletions in the 5′-to-3′ direction. (B) Both *MXR1* and *mxr1* strains carrying the indicated promoter truncations fused to the β-lactamase reporter were grown on glucose to an OD_{600} of 0.5, maintained on glucose or shifted to methanol for 8 h, and harvested. Cell extracts were generated by glass bead lysis and assayed for β-lactamase activity and protein concentration to produce specific activity values. Relative specific activities were indicated as a percentage of the specific activity of the wild-type (WT) strain harboring the pHWG0 vector on methanol induction medium. *AOX2*, region in *P. pastoris* alcohol oxidase II promoter hypothesized by Ohi et al. (35) to be a methanol-regulatory region; K, sequence identified by Kumagai et al. (29) in several promoters that is hypothesized to be a methanol-regulatory region.

TATA box at positions −162 to −155. Each 5′ *AOX1* deletion vector was integrated into the *HIS4* locus of both a wild-type strain (GS115) and an *mxr1* mutant strain (JC132) so that the data generated could be directly compared. The presence of each deletion construct in the genome of the transformed strains was confirmed by colony PCR (data not shown).

Each 5′ *AOX1* deletion strain was grown and sampled in minimal glucose medium and then shifted by centrifugation to methanol medium for 8 h and harvested. Samples were processed into cell extracts and assayed for β-lactamase activity. In addition to β-lactamase, we measured Aox activity to confirm that cultures had induced the methanol pathway normally and assayed for Gap activity to ensure that all glucose-derived extracts were active. Results in strains with a wild-type back-

ground suggested that, as expected, the largest 5′ *AOX1* fragment strongly repressed β-lactamase expression in glucose medium and induced high levels of this reporter in response to methanol. Examination of the deletion series data suggested that methanol-sensitive activation domains exist between positions −735 and −415, −415 and −294, and −294 and −188, since removal of these sequences caused significant decreases in methanol-induced β-lactamase reporter activity (Fig. 6B). Each of these sites appears to be dependent on Mxr1p for activity, since little or no β-lactamase activity was seen in methanol-induced *mxr1* strains harboring the 5′ *AOX1-bla* deletion series. In addition, a glucose-sensitive repression domain appears to exist between positions −294 and −188, since removal of these sequences resulted in a significant increase in the level of β-lactamase in glucose medium. This repression site appeared to function independently of Mxr1p, since the same increase in β-lactamase activity was seen in glucose-grown *mxr1* cells. At this point, our focus was drawn to the *AOX1* upstream region between position −415 and the TATA box (−162) because this 253-bp sequence appeared to contain both repression and activation domains.

We next determined whether the 253-bp sequence, tentatively identified as being necessary for methanol regulation and response to Mxr1p, was sufficient for this regulation and whether it could function properly in a different context from the first. For this, the 5′ *AOX1* deletion fragment from position −415 to position −172 (just 5′ of the *AOX1* TATA box) was inserted into a second test vector, pGC187. The fragment was inserted into this vector just 5′ of a basal transcription unit composed of the *S. cerevisiae LEU2* TATA box and transcription initiation sites. This reporter construct also contained the *LEU2* 5′ untranslated region, and sequences encoding the first 13 amino acids of the *LEU2* product fused in frame to the *E. coli lacZ* gene. In wild-type cells, pGC187, which contained no insert sequences, showed low constitutive expression on either methanol or glucose medium. Insertion of this 243-bp *AOX1* promoter fragment to create pGC182 resulted in *P. pastoris* strains that showed strong levels of β-galactosidase activity in response to methanol but only low levels on glucose, imitating the transcriptional behavior of *AOX1* and of P_{AOX1}-*bla* described above (data not shown). The region of 243 bp located upstream of *AOX1* between its putative TATA box and position −415 appeared to contain critical *cis*-acting elements responsible for *AOX1* transcriptional behavior. Importantly for these studies, this region also was dependent on Mxr1p for proper function, since no induction of β-galactosidase was observed in response to methanol in *mxr1* mutant strains harboring this DNA (data not shown).

Mxr1p binds specifically to sequences upstream of a methanol-regulated gene. The definition of this 243-bp *AOX1* transcriptional regulatory fragment provided us with a key element needed to perform a DNA band shift experiment. The other key element was a source of Mxr1p. Because Mxr1p is produced at only low levels in wild-type *P. pastoris*, it was necessary to construct a *P. pastoris* strain that overexpressed the protein. We first constructed a strain that produced Mxr1p under the control of the strong methanol-regulated *AOX1* promoter with the vector pGC215. However, this strain, which was otherwise the wild type, did not grow on methanol and did not induce Mxr1p or other methanol pathway components in response to methanol. Apparently, high levels of Mxr1p are toxic to *P. pastoris*. As an alternative, we expressed *MXR1* under the control of the promoter from the *P. pastoris PEX8* gene (32). P_{PEX8} is methanol regulated (~5- to 10-fold relative to glucose) but not nearly as strongly induced or repressed as *AOX1*. To aid in visualizing Mxr1p, we also added sequences encoding a C-terminal polyhistidine (His_6) tag and a *myc* epitope to *MXR1*. Finally, we incorporated this P_{PEX8}-*MXR1* construct into a zeocin resistance shuttle vector that facilitated the selection of *P. pastoris* transformants with various copy numbers of the vector inserted into the *P. pastoris* genome. We found that this P_{PEX8}-*MXR1* expression vector, pGC217, rescued our *mxr1*Δ strain with respect to its ability to grow on methanol, demonstrating that our Mxr1p-His_6-*myc* product was fully functional in *P. pastoris*. In addition, we could detect the Mxr1p-His_6-*myc* protein on immunoblots using anti-*myc*. Finally, we screened transformant colonies that were resistant to high levels of zeocin to obtain a collection of strains with a range in the number of copies of the P_{PEX8}-*MXR1* expression vector. A multicopy strain that grew slowly on methanol (due presumably to the deleterious effect of Mxr1p-His_6-*myc* overproduction) but that contained relatively high levels of the product (Fig. 7A) was identified.

Band shift experiments were performed using cell extracts prepared from this Mxr1p overexpression strain and the *mxr1*Δ strain as a negative control. The 243-bp DNA fragment that was shown as described above to confer methanol-regulated expression on reporter genes was labeled. As a negative control, we also labeled a 150-bp fragment from the *AOX1* coding sequence that our studies indicated was not involved in the regulation of *AOX1* transcription. As shown in Fig. 7B, extracts from the Mxr1p-His_6-*myc* overexpression strain were able to shift the *AOX1* promoter fragment, whereas equal amounts of extract from the *mxr1*Δ strain were not. Furthermore, the same amount of extract from either strain did not shift the negative control fragment. Thus, as expected for a DNA-binding transcription factor, Mxr1p specifically bound to one or more sites within the *AOX1* promoter fragment.

Mxr1p is cytoplasmic in glucose-grown cells but localized to the nucleus in cells cultured on methanol or oleate. Finally, we examined the subcellular localization of Mxr1p. A *P. pastoris* strain (SMY175) that expressed Mxr1p with a C-terminal HA epitope tag (Mxr1p-HA) was constructed. The tagged construct, under the control of the *MXR1* promoter, was integrated into the genome of the strain. As a control, the same Mxr1p-HA-expressing construct was shown to be able to restore wild-type methanol and oleate growth rates to an *mxr1* mutant. Thus, the construct was fully functional with regard to Mxr1p. For localization studies, the Mxr1p-HA-expressing strain was cultured on selected carbon sources, and samples of the cultures were prepared for immunofluorescence microscopy using DAPI and antibodies against HA. As shown in Fig. 8, Mxr1p was mostly cytoplasmic in glucose-grown cells, whereas it was mostly localized to a single large compartment in oleate- and methanol-grown cells. This compartment is almost certainly the nucleus, since the same compartment fluoresces with DAPI labeling (see merged Mxr1p-HA and DAPI images in Fig. 8). In further studies with an Mxr1p-green fluorescent protein-expressing *P. pastoris* strain, we observed the same results and in addition observed that Mxr1p is also nu-

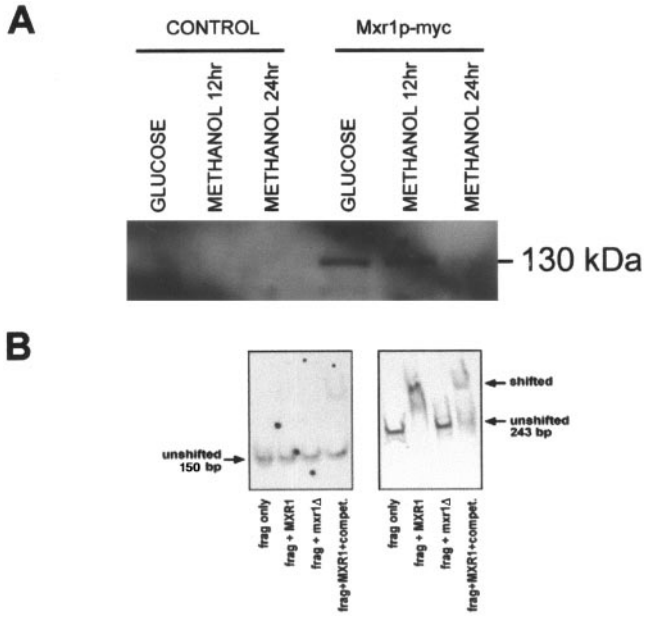


FIG. 7. A strain containing multiple copies of pGC217 (P_{PEX8}-*MXR1*) expresses high levels of Mxr1p, which selectively binds a 243-bp fragment from the *AOX1* promoter. (A) A wild-type strain containing multiple copies of pGC217 as well as a strain containing pJS1 as a negative control were grown in YND medium, spun down, and then resuspended in YNM medium to induce *PEX8* promoter expression. Cell extracts were prepared from the YND culture and from those induced on YNM for 12 and 24 h. Approximately 50 μg of total protein from each sample was run on SDS-PAGE gels, transferred onto nitrocellulose, and probed with anti-*myc* antibodies. (B) DNA fragments of 150 bp (negative control fragment [frag]) or 243 bp (*AOX1* regulatory control fragment from positions −415 to −172) were biotinylated and incubated with equal amounts of cell extract prepared from the *P. pastoris mxr1*Δ or the Mxr1p overexpression strain in the absence or presence of nonlabeled competitor (compet.) fragment. Samples were then separated by SDS-PAGE, and interactions were visualized with autoradiography using the BrightStar BioDetect kit (Ambion, Austin, TX).

clear in ethanol- and glycerol-grown cells. Thus, it appears that Mxr1p is mainly cytoplasmic in glucose but nuclear in cells grown on gluconeogenic carbon sources, including methanol and oleate.

DISCUSSION

When *P. pastoris* cells are shifted from a growth medium containing glucose to medium containing methanol as a sole carbon source, the yeasts undergo a dramatic change in cell architecture. Peroxisomes become much more numerous and prominent. This is in part due to alcohol oxidase (Aox), the first enzyme in the methanol pathway and a resident of the peroxisome matrix, which is strongly induced by methanol and can constitute up to 30% of the total cellular protein. A cellular mechanism must exist by which the entrance of methanol into the cell, coupled with the absence of glucose, is efficiently transduced into the biogenesis of peroxins, methanol pathway enzymes, and other enzymes and proteins, such as those required for mitochondrial respiration and gluconeogenesis, that are needed for the metabolism of this carbon source.

In this work, we have identified Mxr1p as an important part of this transduction process, an essential regulator of multiple genes required for growth on methanol and peroxisome proliferation in response to this substrate. Without *MXR1* function, *P. pastoris* cells do not induce the transcription of many genes involved in methanol metabolism, with *AOX1* being the most dramatically affected. We have provided evidence that Mxr1p is a transcription factor that localizes to the nucleus and binds sequences in the *AOX1* promoter. However, the mechanism of *MXR1* transcriptional activation as well as the signal transduction system that turns on its function remain to be elucidated.

The regulation of genes required for methanol growth in *P. pastoris* has numerous similarities to that of genes for metabolism of the fatty acid oleate in *S. cerevisiae*. Although *S. cerevisiae* does not grow on methanol, it does induce peroxisomes and a peroxisomal fatty acid β-oxidation system in response to oleic acid (28, 48, 56). Also similar to methanol, the *S. cerevisiae* β-oxidation pathway genes appear to be under both a repression/derepression and a carbon source-specific (i.e., fatty acid) induction mechanism. At least two independently functioning regulatory systems have been implicated in the oleate response mechanism in baker's yeast. The first system includes *ADR1*, a DNA-binding (at the upstream activation sequence 1 [UAS1]) transcription factor and the kinase *SNF1* (sucrose nonfermenting 1). Both are considered broad-acting factors involved in the utilization of glycerol, lactate, and ethanol as well as oleate (42). The regulatory circuitries influenced by *SNF1* and *ADR1* show partial overlap; i.e., many but not all *ADR1*-dependent genes also require *SNF1* for expression (60). With regard to growth on oleate medium, strains harboring deletions in either *SNF1* or *ADR1* genes are unable to activate promoters of some but not all genes involved in peroxisome proliferation (44–46).

The second oleate gene regulatory system is comprised of the products of the *PIP2* (peroxisome induction pathway) and *OAF1* (oleate activation factor) genes. These gene products appear to be specific to fatty acid induction and peroxisome biogenesis (27, 28, 37). They function as a heterodimer and bind oleate response elements found in the promoters of genes encoding many peroxins and peroxisomal enzymes. Both Δ*pip2* and Δ*oaf1* strains are unable to induce most *PEX* genes and β-oxidation pathway genes and contain small, less-numerous peroxisomes relative to wild-type, oleate-induced cells.

Two major connections have been established in the area of peroxisome induction between the more general-acting Adr1p and the more specific-acting Pip2/Oaf1 complex. First, overlapping oleate response elements and UAS1 sequence elements have been found in the promoters of several genes required for peroxisome biogenesis. These sequence elements have been shown to be bound coordinately by Pip2p/Oaf1p and Adr1p, respectively, to activate transcription under oleate growth conditions (22, 23). Second, Adr1p has been implicated in the binding and activation of the *PIP2* promoter (38). These findings support a model whereby Snf1p posttranslationally activates Adr1p, which induces the transcription of *PIP2* and coordinates with the Oaf1p/Pip2p complex to directly activate the transcription of a subset of genes (60, 61). Thus, although a complex signaling network must modulate the response to oleate in *S. cerevisiae* cells, the Adr1 protein seems to act at both global and local levels to activate transcription of a wide

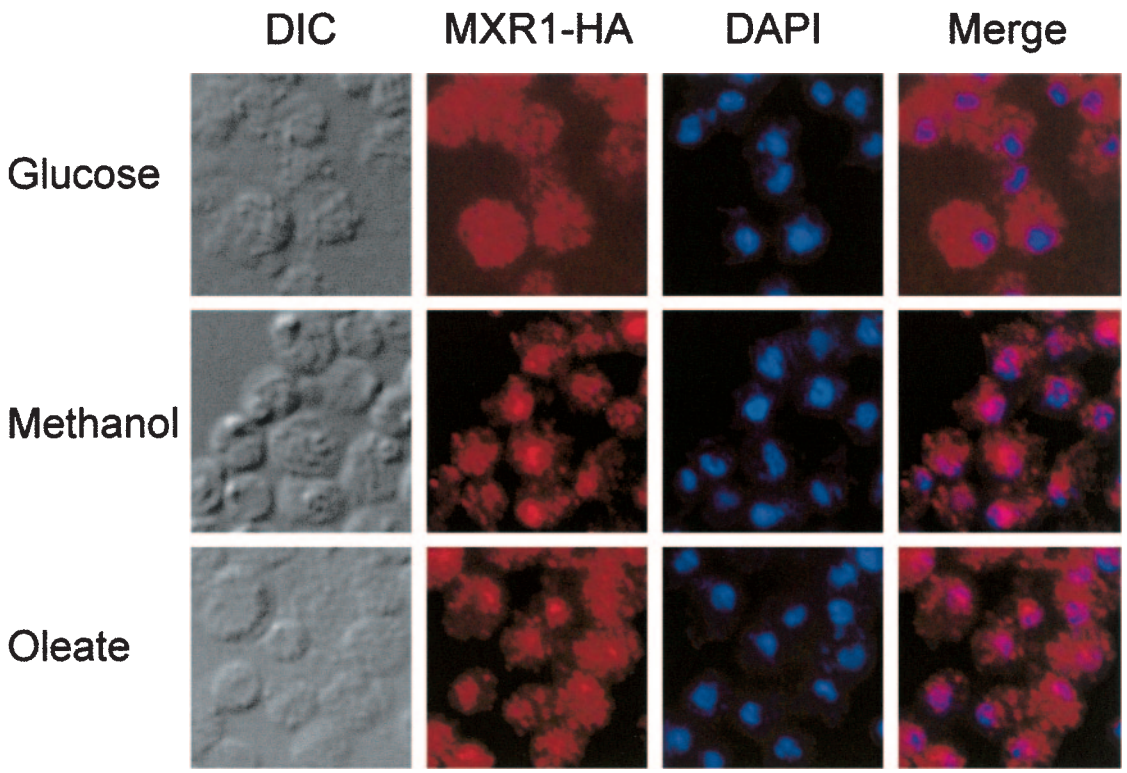


FIG. 8. Mxr1p is cytoplasmic in glucose-grown cells but localized to the nucleus in methanol- or oleate-grown cells. Cultures of *P. pastoris* strain SMY175 were precultured in YPD medium and then shifted by centrifugation to a minimal medium with glucose, methanol, or oleic acid as a carbon source. Samples of these cultures were then harvested and processed for immunofluorescence microscopy. DIC, differential interference contrast.

range of target genes involved in the metabolism of a variety of alternative carbon sources, while Pip2p and Oaf1p appear to regulate a smaller subset of genes involved in oleate metabolism and peroxisome proliferation.

P. pastoris Mxr1p has a number of functional similarities to *S. cerevisiae* Adr1p. Like Adr1p, Mxr1p is necessary for the activation of a number of genes on more than one carbon source and is directly involved in binding the promoter of at least one of these target genes. Mutants in *ADR1* display phenotypes that are strikingly similar to that of the *P. pastoris mxr1* mutant (44, 46). Both mutant strains of yeast cannot grow on oleate medium. Adr1p is required for growth on a number of gluconeogenic carbon sources, while Mxr1p function appears more specific to carbon sources requiring peroxisomes. However, *mxr1* mutants also display a reduced growth rate on many other gluconeogenic carbon sources, suggesting that Mxr1p is involved to some degree in the metabolism of all these carbon sources. Both *MXR1* and *ADR1* are constitutively expressed at a low level on all carbon sources, suggesting some type of activation by a posttranslational mechanism (47).

The localization of Mxr1p to the nucleus under gluconeogenic conditions, including methanol and oleic acid, is not surprising, given the probable function of the protein as a transcription factor, although how it reaches this location is not clear. A search of the amino acid sequence of Mxr1p using PSORT and PS software did not reveal a potential nuclear targeting signal in the protein. Possibly, the protein contains an unknown targeting signal or becomes nuclear upon dimerization with another nuclear localization signal-containing protein. Also, the movement of Mxr1p from the cytoplasm to the nucleus is not surprising. An analogous phenomenon occurs with many transcription factors, including the major transcriptional repression factor Mig1p, which moves in the opposite direction as Mxr1p from the nucleus in glucose-grown cells to the cytoplasm under gluconeogenic conditions (16). Interestingly, Adr1p is different in this respect, as it is believed to contain a nuclear localization signal and to be nuclear under all growth conditions (47).

In addition to these functional similarities, the Adr1 and Mxr1 proteins share common sequence features, most strikingly in the ~70-amino-acid region near their N termini. Mxr1p and Adr1p each contain two C_2H_2-type zinc fingers in this region with 70% identity and 82% similarity. Especially striking is the fact that all residues in this region that are critical for Adr1p to recognize and bind to UAS1 are identical in Mxr1p (50, 51). Other residues proximal to the zinc finger regions that play a role in zinc finger conformation and stability are also highly conserved between the two proteins (8). Other similarities between Mxr1p and Adr1p include three potential phosphorylation sites. The potential cAMP-dependent protein kinase phosphorylation site of Adr1p (serine 230) has been shown to be important for the optimal functioning of Adr1p (8, 15, 50). Mxr1p contains a nearly identical potential cAMP-dependent protein kinase phosphorylation sequence (near serine 220) in approximately the same location relative to its zinc fingers. Two other potential protein kinase C phosphory-

lation sites located within or near the zinc finger domains are also common to the two proteins (47). Finally, within the 243-bp *AOX1* promoter fragment shown to be bound by extracts overexpressing Mxr1p is a region that conforms to the consensus sequence for UAS1, to which Adr1p binds (52). Although the amino acid similarities seen outside of the zinc finger region are more modest, the functional and structural similarities between *ADR1* and *MXR1* are striking.

However, there are also a number of clear differences between *S. cerevisiae adr1* and *P. pastoris mxr1* strains. *adr1* mutants cannot utilize oleate because they are unable to transcriptionally activate genes involved in fatty acid metabolism. However, the expression of several genes required for oleate utilization, examined at both the protein and mRNA levels, appears normal in *mxr1* mutants. We are still searching for a reason why *mxr1* strains cannot grow on oleate. It is reasonable to assume that Mxr1p must affect some aspect of oleate metabolism that has not yet been tested. Moreover, the finding that the induction of the *PEX14* promoter is greatly reduced in *mxr1* strains in methanol medium but only slightly affected in oleate medium suggests a complex situation regarding its transcriptional modulation. The activation of the same target promoter under methanol and oleate growth conditions must occur by different mechanisms. Another difference is that *S. cerevisiae ADR1* is absolutely required for growth on ethanol and glycerol, whereas *P. pastoris* growth on these carbon sources is only modestly affected in *mxr1* mutants.

We propose that *MXR1* is the *P. pastoris* homologue of the *S. cerevisiae ADR1* gene but that it has changed ("rewired") through evolution with regard to the spectrum of genes under its control. These changes could most easily be explained as the evolution of new UASs in promoters of genes added to its control and the loss of UASs from genes it no longer regulates. Two pieces of evidence support this conjecture. First, a search of the *P. pastoris* genomic sequence shows that of all predicted *P. pastoris* proteins, Mxr1p is clearly the closest in sequence similarity to Adr1p. Second, the function of these two transcription factors in regulating genes involved in the metabolism of peroxisomal carbon sources is similar. Thus, the evolution of one to another would presumably require the "rewiring" of promoters for a relatively small subset of genes.

Because Mxr1p modulates the expression of several genes (i.e., *PEX8*, *PEX14*, and *FLD1*) involved in peroxisome biogenesis in response to methanol, we are currently examining their promoters for elements homologous to those responsible for methanol activation in the *AOX1* promoter. In addition, we are continuing to focus our efforts on the identification of other *trans*-acting factors that regulate the expression of proteins involved in oleate and methanol utilization by using both genetic and homology-based approaches (26). This effort should become easier when the sequencing of the *P. pastoris* genome is completed (which is expected to happen in 2006). In addition, work on other methylotrophic yeasts should also identify factors involved in this process. Several methanol utilization-defective mutants of *Hansenula polymorpha* that display reduced methanol oxidase (*MOX*) promoter activity have been identified (54). Most recently, a transcription factor, known as *MPP1*, that regulates peroxisomal proliferation under methanol-induced conditions has been identified in *H. polymorpha* (30). In cells lacking Mpp1p function, matrix enzymes involved in methanol metabolism, such as alcohol oxidase, and peroxins are present at reduced levels compared to those of the wild type, preventing the cells from growing on this carbon source. Although its primary sequence differs greatly from that of Mxr1p, the similarities between the *mxr1* and *mpp1* phenotypes suggest that a conserved regulatory mechanism may control peroxisome proliferation in the various species of methylotrophic yeasts.

Because *P. pastoris* is a major system for the production of recombinant proteins, an understanding of the factors that regulate methanol induction will further our comprehension of heterologous protein expression in this yeast. The characterization and, ultimately, the optimization of methanol-induced transcription could potentially improve its productivity as a host for important recombinant proteins needed for the treatment of diseases.

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