Regulation of Acetate Metabolism and Acetyl Co-a Synthetase 1 (ACS1) Expression by Methanol Expression Regulator 1 (Mxr1p) in the Methylotrophic Yeast Pichia pastoris*

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Methanol expression regulator 1 (Mxr1p) is a zinc finger protein that regulates the expression of genes encoding enzymes of the methanol utilization pathway in the methylotrophic yeast Pichia pastoris by binding to Mxr1p response elements (MXREs) present in their promoters. Here we demonstrate that Mxr1p is a key regulator of acetate metabolism as well. Mxr1p is cytosolic in cells cultured in minimal medium containing a yeast nitrogen base, ammonium sulfate, and acetate (YNBA) but localizes to the nucleus of cells cultured in YNBA supplemented with glutamate or casamino acids as well as nutrient-rich medium containing yeast extract, peptone, and acetate (YPA). Deletion of Mxr1 retards the growth of P. pastoris cultured in YNBA supplemented with casamino acids as well as YPA. Mxr1p is a key regulator of ACS1 encoding acetyl-CoA synthetase in cells cultured in YPA. A truncated Mxr1p comprising 400 N-terminal amino acids activates ACS1 expression and enhances growth, indicating a crucial role for the N-terminal activation domain during acetate metabolism. The serine 215 residue, which is known to regulate the expression of Mxr1p-activated genes in a carbon source-dependent manner, has no role in the Mxr1p-mediated activation of ACS1 expression. The ACS1 promoter contains an Mxr1p response unit (MxRU) comprising two MXREs separated by a 30-bp spacer. Mutations that abrogate MxRU function in vivo abolish Mxr1p binding to MxRU in vitro. Mxr1p-dependent activation of ACS1 expression is most efficient in cells cultured in YPA. The fact that MXREs are conserved in genes outside of the methanol utilization pathway suggests that Mxr1p may be a key regulator of multiple metabolic pathways in P. pastoris.

The ability of yeast cells to grow in the presence of diverse carbon sources offers a unique opportunity to study various metabolic pathways, which is not always feasible in higher eukaryotic systems. In addition to glucose, yeast cells can utilize acetate, ethanol, glycerol, or fatty acids as the sole source of carbon, and the study of their metabolism and regulation has been one of the fascinating areas of biochemistry. The regulation of metabolic pathways of respiratory yeasts such as Pichia pastoris has not been as well studied as that of Saccharomyces cerevisiae despite the extensive use of the former for the commercial production of recombinant proteins. P. pastoris, a methylotrophic yeast, can metabolize a number of compounds, such as glycerol, methanol, acetate, and oleic acid, in addition to glucose. However, very little information is available on the transcriptional regulation of metabolic pathways other than the methanol utilization (mut)2 pathway in this yeast species. The expression of genes of the mut pathway is regulated by at least three zinc finger proteins (1–6). Of these, methanol expression regulator 1 (Mxr1p) activates the expression of genes of the mut pathway by binding to Mxr1p response elements (MXREs) in their promoters (2, 3). Rop1p has the same DNA binding specificity as Mxr1p and functions as a repressor of genes of the mut pathway in P. pastoris cultured in nutrient-rich medium containing yeast extract, peptone, and methanol (YPM) but not minimal medium containing a yeast nitrogen base, ammonium sulfate, and methanol (YNBM) (4, 5). Tim1p is also essential for the expression of genes of the mut pathway (6). However, its mechanism of action remains unknown. The differential regulation of methanol metabolism in YNBM and YPM by Mxr1p and Rop1p led us to investigate the transcriptional regulation of other metabolic pathways in cells cultured in minimal and nutrient-rich media. In this study, we demonstrate that Mxr1p regulates acetate metabolism only in cells cultured in nutrient-rich medium containing yeast extract, peptone, and acetate (YPA) but not minimal medium containing yeast nitrogen base, ammonium sulfate and acetate (YNBA).

Experimental Procedures

Yeast and Bacterial Strains—P. pastoris (GS115, his+) was cultured in either nutrient-rich medium (1.0% yeast extract and 2.0% peptone) containing 2.0% glucose (YPD) or 2.0% acetate (YPA) or minimal yeast nitrogen base medium (0.17% yeast nitrogen base without amino acids and 0.5% ammonium sulfate) supplemented with 2.0% glucose (YNBD) or 2.0% sodium acetate (YNBA). Casamino acids (CAAs) and glutamate were added to YNBA medium to final concentrations of 1.0% and 0.5%, respectively. P. pastoris strains were grown at 30 °C in an

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2 The abbreviations used are: mut, methanol utilization pathway; MXRE, Mxr1p response element; YPM, yeast extract, peptone, and methanol; YNBM, yeast nitrogen base, ammonium sulfate, and methanol; YNBA, yeast nitrogen base, ammonium sulfate, and acetate; CAA, casamino acid; qPCR, quantitative PCR; TRITC, tetramethylrhodamine isothiocyanate; Glu, glutamate; ACS, acetyl-CoA synthetase; MxRU, Mxr1p response unit; PGK, phosphoglycerate kinase; YPA, yeast extract, peptone, and acetate.
orbital shaker at 180 rpm. For all growth and β-galactosidase assays, colonies were first cultured overnight in YNB medium supplemented with histidine, washed with sterile water, and shifted to the respective media with an initial optical density of ~0.1. *Escherichia coli* DH10β and BL21 (DE3) strains were used for plasmid isolation and recombinant protein expression, respectively. Bacterial and yeast transformations were done by electroporation (Gene Pulser, Bio-Rad) according to the instructions of the manufacturer.

**Antibodies and Other Reagents**—Oligonucleotides were purchased from Sigma-Aldrich (Bangalore, India). Anti-His tag, anti-c-myc tag, and anti-FLAG tag antibodies were purchased from Thermo Scientific (Bangalore, India), Merck Millipore (Bangalore, India), and Sigma-Aldrich, respectively.

**Construction of the *P. pastoris* ∆mxr1 Strain**—In the ∆mxr1 strain, *Mxr1* encoding 320 N-terminal amino acids was replaced by a zeocin expression cassette. This deletion construct was generated by four different PCR reactions using *P. pastoris* genomic DNA and the pGAPZA vector (Invitrogen) as templates as well as a series of overlapping and non-overlapping primers. To begin with, the *Mxr1* promoter (~997 to ~1 bp) was amplified from *P. pastoris* genomic DNA using primer pair 1F (5′-GTGGAATCTATACCC CTCTCTC-3′, ~997 to ~970 bp of the *Mxr1* promoter) and 1R (5′-GCTA TGTTCTGTTGAGGATCCGCATG-3′, ~970 to ~961 bp of the pGAPZA vector (uppercase) and ~1 to ~25 bp of the *Mxr1* promoter (lowercase)). In another PCR reaction, a 1.2-kb region of the *pGAPZA* vector (1419–2591 bp) was amplified from *P. pastoris* genomic DNA using primer pair 2F (5′-AGTAGCCATTCAAgctcacatgttggtctccagcttg-3′) and 2R (5′-TCACACACCATAGCGCC-3′), which amplifies a region between +1 and +435 bp of *Mxr1*. This PCR product was then radiolabeled and used as probe A in Southern blotting. Probe B for Southern blotting was generated by PCR amplification of a region between +1201 and +1250 bp of *Mxr1* using the primer pair P3 (5′-ACCTTCTTAATGCCCACATTTGGC-3′) and P4 (5′-TAAAGAAC GGTGGTGAATGAACTC-3′). For Southern blotting, genomic DNA was digested with PstI and BamHI.

**Construction of *P. pastoris* Expressing Chromosomally FLAG-tagged *Mxr1* (Pmxf1PFLAG)**—A zeocin resistance expression cassette fused to the gene encoding FLAG-tagged *Mxr1* was obtained by five different PCR reactions. First, a 1.2-kb zeocin expression cassette (1419–2591 bp) was amplified from the *pGAPZA* vector using primers pair 1F (5′-TGCGGATCCCC CACACACCATAGC-3′, 962–986 bp of the *pGAPZA* vector) and 1R (5′-GCTATAGATAAGATCCACAA TTTCTCAATGCCATGTTGCTG-3′, ~1007 to ~979 bp of the *Mxr1* promoter (uppercase) and 2161–2136 bp of the *pGAPZA* vector (lowercase)). In another PCR reaction, 1007 bp of *Mxr1* encompassing ~1007 to ~1 bp were amplified from *P. pastoris* genomic DNA using the primer pair 2F (5′-CAAGCTGGGAC ACAAATGTCGAATTgagaaaaattggtctcatcattag-3′, 2161–2136 bp of the *pGAPZA* vector (uppercase) and ~1007 to ~979 bp of *Mxr1* (lowercase)) and 2R (5′-CGTCATGCTTCA TTGTA GCCTCATGTTGCTG-3′, ~928–953 bp encoding a 3× FLAG tag (uppercase) and ~25 to ~1 bp of *Mxr1* (lowercase)). In the third PCR reaction, a 72-bp sequence encoding a 3× FLAG tag was amplified from the 3× FLAG vector (Sigma-Aldrich) using the primer pair 3F (5′-GTTGGAATCTATACA CCAATGTCGAATTgagaaaaattggtctcatcattag-3′, ~25 to ~1 bp of *Mxr1* (uppercase) and 928–953 bp encoding a FLAG tag (lowercase)) and 3R (5′-GACACAAAGTTG GGGTAGATTTGCTcttgctcatgcattgtaatc-3′, +4 to +23 bp of *Mxr1* (uppercase) and 976–1000 bp encoding a FLAG tag (lowercase)). In the fourth PCR reaction, the gene encoding 797 N-terminal amino acids of *Mxr1* was amplified using the primer pair 4F (5′-GTTGGAATCTATACCCCTCTCTC-3′) and 4R (5′-TTATCCAAAGGTTTG TTGATTTGAAAAAG-3′, 1942–1970 bp of *Mxr1*). All three PCR products containing overlapping regions were pooled (50 ng each) and used as templates in a final PCR reaction along with primers 1F and 3R. The 3.2-kb PCR product thus obtained was transformed into the *P. pastoris* GS115 strain to generate the zeocin-resistant *Δmxr1* strain, in which the region encoding the 320 N-terminal amino acids of *Mxr1* was replaced by a zeocin expression cassette.

**Characterization of the *P. pastoris* Δmxr1 Strain**—Deletion of the region encoding the DNA binding domain was confirmed by PCR as well as Southern blotting. PCR was carried out with genomic DNA as a template and the primer pair P1 (5′-ATGAGCAATCTACCC CAAC-3′) and P2 (5′-GCGGG CGCTTCTGAACCTTTCG-3′), which amplifies a region between +1 and +435 bp of *Mxr1*. This PCR product was then radiolabeled and used as probe A in Southern blotting. Probe B

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**Regulation of Acetate Metabolism in Pichia pastoris**

**Construction of *P. pastoris* Strains Overexpressing *Mxr1*, *Adr1*, *Mxr1*^NADH^, and *Mxr1*^S215A^**—For overexpression of *Mxr1* and *Adr1*, the pGAPBA vector containing a basicidin resistance gene was generated by PCR amplification of the basicidin resistance expression cassette from the pPlc6 vector (Life Technologies) using the primer pair 5′-TGCGGATCCC CCACACACCATAGC-3′ and 5′-CTCATGTTGCTT CCACCTTG-3′ and cloning it into the Smal site of the *pGAPZA* vector.
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The gene encoding full-length Mxr1p was obtained by PCR amplification of *P. pastoris* (GS115) genomic DNA using the primer pair 5’-CGGGTTACCAATGAGCAATCTACCCCCAAC-3’ and 5’-ATAAGAATGCGGCCGACACACACCACTTACATCCCCAAC-3’. Similarly, the gene encoding full-length Adr1p was amplified from *S. cerevisiae* genomic DNA using the primer pair 5’-CGGGGTACCATGGCTAACGTAGAAAAA-3’ and 5’-ATAAGAATGCGGCCGACACACACCACTTACATCCCCAAC-3’. The KpnI and NotI restriction sites are underlined. Following restriction digestion with KpnI and NotI, the PCR product was cloned into the pGAPMxl vector and pGAPAdr1 vectors, respectively.

The pGAPMxr1 vector overexpressing 400 N-terminal amino acids of Mxr1p was generated by PCR amplification of the *Mxr1* gene encoding 400 N-terminal amino acids of Mxr1p from *P. pastoris* genomic DNA using the primer pair 5’-CGGGTTACCAATGAGCAATCTACCCCCAAC-3’ and 5’-ATAAGAATGCGGCCGACACACACCACTTACATCCCCAAC-3’. Similarly, the gene encoding full-length Adr1p was amplified from *S. cerevisiae* genomic DNA using the primer pair 5’-CGGGGTACCATGGCTAACGTAGAAAAA-3’ and 5’-ATAAGAATGCGGCCGACACACACCACTTACATCCCCAAC-3’. 

The S215A mutant of Mxr1p was generated by site-directed mutagenesis using the QuikChange method (Stratagene) using primer pairs 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ and 5’-ATAAGAATGCGGCCGACACACACCACTTACATCCCCAAC-3’. The KpnI and NotI restriction sites are underlined. Following restriction digestion with KpnI and NotI, the Mxr1 and Adr1 genes were cloned into the KpnI- and NotI-digested pGAPMxr1 vector to obtain the pGAPMxr1 vector.

The expression of histidine-tagged ACS1 (ACS1His) was examined by Western blotting using anti-His tag antibodies.

Expression of β-Gal from the ACS1 Promoter (pACS)—pACS-lacZ constructs consisting of the *E. coli lacZ* gene downstream of pACS were generated in three PCR reactions. pACS-lacZ, consisting of 1049 bp of pACS upstream of lacZ, was generated by PCR using *P. pastoris* genomic DNA as a template and the primer pair 1F (5’-CGCGGATCCCAAAAAACACCCAGCTGTGAGAG-3’, 1049 to 1027 bp of pACS1) and 1R (5’-CGGGTTAAGAACGAGGGCATATTAGGATAAAGGATCTG-3’, 20 to 1 bp of pACS1 (upper case) and +1 to +25 bp of the *E. coli lacZ gene* (lower case)). The BamHI site is underlined. In the second PCR reaction, the *E. coli lacZ* gene was amplified from pFRT/lacZeo (Life Technologies) using the primer pair 2F (5’-GATACGACTTATGTTGATCTGACATTGCTGTTTGAAC-3’, 25 to 1 bp of pACS1 (upper case) and +1 to +20 bp of lacZ (lower case)) and 2R (5’-CCCAACTTGATCGTGTCGTCGTCGTCGTTAGGTTTTCCAAG-3’, 3491 to +3510 bp of lacZ). The HindIII site is underlined. In the third PCR reaction, the PCR products from the first two reactions were used as templates and amplified using the 1F and 2R primers to get the pACSlacZ expression cassette, which was digested with BamHI and HindIII and cloned into pb3 (Addgene) to generate pb3-pACS1-lacZ.

To generate pb3-pACS1-lacZ containing 809 bp of pACS upstream of lacZ, a PCR reaction was carried out using pb3-pACS1-lacZ as a template and the primer pair 3F (5’-CGCGGATCCCAAAAAACACCCAGCTGTGAGAG-3’, 1049 to 1027 bp of pACS1) and 2R. To generate pb3-pACS3-lacZ, consisting of 736 bp of pACS upstream of lacZ, a PCR reaction was carried out using the primer pair 4F (5’-CGCGGATCCCAAAAAACACCCAGCTGTGAGAG-3’, 1049 to 1 bp of pACS1 (upper case) and +1 to +20 bp of lacZ (lower case)) and 2R (5’-CCAAGCTTGATCGTGTCGTCGTCGTTAGGTTTTCCAAG-3’, 3491 to +3510 bp of lacZ). The HindIII site is underlined. In the third PCR reaction, the PCR products from the first two reactions were used as templates and amplified using the 1F and 2R primers to get the pACSlacZ expression cassette, which was digested with BamHI and HindIII and cloned into pb3 (Addgene) to generate pb3-pACS1-lacZ.

Mutations were introduced into the MXRs of pACS using the QuikChange method (Stratagene) using primer pairs carrying appropriate mutations. The primer pair 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ and 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ was used to generate pb3-pACS1-M1-lacZ. pb3-pACS1-lacZ was used as the template for PCR. The primer pair 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ and 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ was used to generate pb3-pACS1-M2-lacZ. pb3-pACS1-lacZ was used as the template for PCR. The primer pair 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ and 5’-GACAGCACAATCGTATCACCACTTACATCCAGAAGAAAAC-3’ was used to generate pb3-pACS1-M3-lacZ. pb3-pACS1-M2-lacZ was used as the template. Mutations are underlined.
A 10-bp sequence (5’-ccaaacatc-3’) was inserted between the two MXREs of pACS1-lacZ as follows. The PCR reaction was carried out with P. pastoris genomic DNA as a template and the primers i10F1 (5’-CGCGGATCCAAAACCAAGCTAGC-GTACAGG-3’ (−1049 to −1027 bp of pACS1)) and i10R1 (5’-GAAGAGAAGATAGTGTTCGAGGATGCC-3’). The BamHI site is underlined. In the second PCR reaction, the pACS1 promoter along with the lacZ gene was amplified from plB3-pACS1-lacZ using the primers i10F2 (5’-GTATCCCCCTCTCTCGTAAAGGACAAAC-accaaaacatcccaaaacatc-3’) and i10R2 (5’-CCCAAGCTTCTAGTGTTTGCGGCC-3’). The BamHI and HindIII sites are underlined. The 10-bp insertion sequence in the primers is shown in lowercase. In the third and final PCR reaction, the PCR products of first and second PCR reactions were used as templates along with the i10F1 and i10R2 primer pair. The PCR product was digested with BamHI and HindIII and cloned into the plB3 vector to obtain plB3-pACS1(i10)-lacZ.

A 20-bp sequence (5’-ccaaacatcccaaaacatc-3’) was inserted between the two MXREs of pACS1-lacZ as follows. The PCR reaction was carried out with P. pastoris genomic DNA as a template and the primers i10F1 (5’-CGCGGATCCAAAACCAAGCTAGCTACAGAG-3’ (−1049 to −1027 bp of pACS1)) and i20R1 (5’-GAAGAGAAGAAATGATGTTTGCGGCC-3’). The BamHI and HindIII sites are underlined. The 20-bp insertion sequence in the primers is shown in lowercase. In the third and final PCR reaction, the PCR products of first and second PCR reactions were used as templates along with the i10F1 and i10R2 primer pair. The PCR product was digested with BamHI and HindIII and cloned into the plB3 vector to obtain plB3-pACS1(i20)-lacZ. Recombinant plasmids were transformed into GS115 and MXR1 strains and plated on YNBD-His+G418 (5). After transformation, the plasmids were recovered from the transformants by the Ncol restriction site in the primers was underlined. The 10-bp insertion sequence in the primers is shown in lowercase. In third and final PCR reaction, the PCR products of first and second PCR reactions were used as templates along with the i10F1 and i10R2 primer pair. The PCR product was digested with BamHI and HindIII and cloned into the plB3 vector to obtain plB3-pACS1(i10)-lacZ. Recombinant plasmids were transformed into GS115 and MXR1 strains and plated on YNBD-His−agar plates, and β-galactosidase assays were carried out with three individual colonies for each transformant essentially as described previously (7).

DNA-Protein Interactions—Recombinant Mxr1pN130 was purified from E. coli extracts, and its ability to bind to radiolabeled oligonucleotides containing pACS-MXREs was examined by EMSA essentially as described previously (7).

Mxr1 encoding 400 N-terminal amino acids was expressed as a GST fusion protein (Mxr1pN400) in E. coli by PCR amplification of Mxr1pN400 using the primer pair 5’-CGCGGATCCCAT-GAGAATCTACCCCACACT-3’ and 5’-TAAGCTGACCGCGGACGATGACATAGTTAGAGAAAAG-3’ and cloning into the pGEX4T1 vector (GE Healthcare). The BamHI and NotI restriction sites in the primers are underlined. The recombinant plasmid was transformed into the E. coli BL21(DE3)pLYsS strain, and recombinant protein expression was induced by the addition of 1 mM isopropyl-1-thio-β-D-galactopyranoside. Mxr1pN400 was purified by glutathione affinity chromatography according to the instructions of the manufacturer (GE Healthcare).

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Subcellular Localization of FLAG-Mxr1p—Subcellular localization of FLAG-Mxr1p in P. pastoris cells cultured in YNBA and YPA was examined by immunofluorescence using a fluorescence microscope (Leica). Mouse anti-FLAG antibodies and TRITC-conjugated rabbit anti-mouse antibodies were used. Immunofluorescence was carried out essentially as described previously (5).

Northern Blotting Analysis and Quantitative Real-time PCR—RNA isolation, semiquantitative PCR, and Northern blotting analysis were carried out as described previously (5). Real-time PCR was performed using iQ SYBR Green Super Mix and a q5 multicolor real-time PCR thermal cycler (iCycler, Bio-Rad). The levels of mRNA expression in Δmxr1 and P. pastoris strains overexpressing Mxr1p or Mxr1pN400 relative to GS115 were normalized to tubulin mRNA. Data were analyzed by the comparative Ct method for relative quantification (ΔΔCt method), which describes the change in expression of the target genes in a test sample relative to a calibrator sample.

Results

Regulation of Acetate Metabolism by Mxr1p in P. pastoris Cultured in YPA Medium—To examine whether Mxr1p has a role in the regulation of acetate metabolism, we generated a Δmxr1 strain in which the 320 N-terminal amino acids of Mxr1p, including the zinc finger DNA binding domain, were replaced by a zeocin expression cassette (Fig. 1A). The deletion was confirmed by PCR using primers against the DNA binding domain of Mxr1p from genomic DNA (Fig. 1B) and by Southern blotting analysis using 32P-labeled probes A and B, which hybridize to regions within and outside of the region encoding the DNA binding domain, respectively (Fig. 1C). The Δmxr1 strain was unable to grow in YNBM (Fig. 1D) as expected. Deletion of Mxr1p had no effect on the growth of P. pastoris cultured in minimal medium, YNBA medium, and YNBA medium supplemented with 0.5% glutamate (YNBA + Glu) (Fig. 1, E and F). However, Δmxr1 exhibited impaired growth when cultured in YNBA medium supplemented with 1% casamino acids (YNBA + CAA) and nutrient-rich YPA medium (Fig. 1, G and H). To understand the function of Mxr1p during acetate metabolism, we examined the expression of key genes involved in acetate metabolism, such as ACS1 and ACS2, encoding acetyl-CoA synthetase (ACS), which catalyzes the first step in acetate metabolism as well as ACCH encoding acetyl-CoA hydratase catalyzing the conversion of acetyl-CoA to acetate (Fig. 1D). Northern blotting analysis revealed that Mxr1p activates the expression of ACS1, but not ACS2 and ACCH, in cells cultured in YPA medium (Fig. 1). A P. pastoris strain expressing genomically FLAG-tagged Mxr1p (Pp-Mxr1pFLAG) from its own promoter was generated, and subcellular localization of Mxr1pFLAG was studied in cells cultured in YNBA, YPA, YNBA + CAA, or YNBA + Glu media using anti-FLAG antibodies. Mxr1pFLAG was cytoplasmic in cells cultured in YNBA medium but localized to the nucleus of cells cultured in YPA, YNBA + CAA, and YNBA +...
Glu media (Fig. 1, K and L). Although Mxr1p was localized prominently in the nucleus of almost all cells cultured in YPA medium, nuclear localization of Mxr1p was highly variable among cells cultured in YNBA + CAA or YNBA + Glu media (Fig. 1L).

**Overexpression of Mxr1p, but Not Adr1p, Enhances ACS1 Expression and Growth of P. pastoris**—To confirm the role of Mxr1p in the regulation of ACS1 expression, the *Pp-Mxr1-OE* strain was generated, in which Mxr1 was overexpressed as myc-tagged protein (Mxr1p^{MYC}) from the GAPDH promoter.
Mxr1pMyc overexpressed from the GAPDH promoter was present at much higher levels than Mxr1pFLAG expressed from its own promoter, as evident from Western blotting analysis (Fig. 2, A and B). The mRNA levels of Mxr1Myc were also higher than that of Mxr1FLAG, as evident from qPCR (Fig. 2C). We also generated the Pp-Adr1-OE strain to examine the ability of S. cerevisiae Adr1 encoding Adr1p (alcohol dehydrogenase II synthesis regulator) to complement Mxr1p function because Adr1p is considered to be a homologue of Mxr1p (1, 2, 8, 9). Expression of Myc-tagged Adr1p (Adr1pMyc) from the GAPDH promoter was confirmed by Western blotting using anti-c-myc antibodies (Fig. 2D) as well as semiquantitative RT-PCR (Fig. 2E). Overexpression of Mxr1p, but not Adr1p, results in a significant increase in ACS1 mRNA, as evident from Northern blotting analysis (Fig. 2F). Mxr1 overexpression results in a much higher level of ACS1His than in GS115, as evident from Western blotting (Fig. 2, G and H). An Mxr1p-mediated increase in ACS1 expression results in a significant increase in the rate of growth of P. pastoris in YPA medium (Fig. 2I). Furthermore, an Mxr1p-mediated increase in ACS1His was not observed in cells cultured in YPG medium containing glycerol as a carbon source (Fig. 2J), indicating that the presence of acetate in the nutrient-rich medium is essential for Mxr1p-mediated activation of ACS1 expression.

The N-terminal region of Mxr1p contains the zinc finger domain, the 14-3-3 protein interaction region, and a trans-activation domain (Fig. 3A) (9). To examine the role of the N-terminal trans-activation domain in the regulation of ACS1 expression and acetate metabolism, we overexpressed Mxr1pN400 in Δmxr1 and generated the Pp-Mxr1pN400-OE strain. Overexpression of Mxr1N400 was confirmed by qPCR (Fig. 3B), and protein expression was confirmed by Western blotting using anti-c-myc antibodies (Fig. 3C). Overexpression of Mxr1pN400 results in up-regulation of ACS1, resulting in an increase in ACS1 mRNA (Fig. 3D) and in protein levels (Fig. 3, E and F), leading to an increase in growth rate (Fig. 3G).
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We generated Mxr1pS215A and expressed it in the pathway when cultured in a medium containing ethanol (9). The interaction is abrogated, resulting in derepression of genes of the mut pathway expressing the S215A mutant, Mxr1p-14-3-3 protein interaction, in which serine 215 of Mxr1p has a key role (9). In cells repressed because of the interaction of Mxr1p with the 14-3-3 protein, in which serine 215 of Mxr1p has a key role (9). In cells repressed because of the interaction of Mxr1p with the 14-3-3 protein, in which serine 215 of Mxr1p has a key role (9).

To identify cis-acting elements in pACS-mRNA and ACS His protein levels are comparable between Pp-Mxr1-OE and Pp-Mxr1S215A-OE (Fig. 3, I–K). Overexpression of Mxr1pS215A results in slight derepression, resulting in an ~4-fold increase in AOX1 mRNA in cells cultured in YPA (Fig. 3L). This increase is not significant because AOX1 expression in GS115 cultured in YPM is several hundredfold higher than that observed in Pp-Mxr1S215A-OE cultured in YPA (Fig. 3M).

Identification and Characterization of the MxRU in pACS—To identify cis-acting elements in pACS involved in Mxr1p-mediated regulation of ACS1 expression, −1049 bp, −809 bp, −736 bp, and −449 bp of pACS were cloned upstream of E. coli lacZ encoding β-galactosidase to generate the pACS1-lacZ, pACS2-lacZ, pACS3-lacZ, and pACS4-lacZ plasmids, respectively (Fig. 4A). These constructs were introduced into GS115 as well as Δmxr1, and β-galactosidase activity was measured in cells cultured in YPA and YNBA. Mxr1p-dependent activation of pACS was observed in the cases of pACS1-lacZ and pACS2-lacZ, but not pACS3-lacZ and pACS4-lacZ, in cells cultured in YPA but not YNBA (Fig. 4B), indicating that the region between −809 and −736 bp is essential for Mxr1p function. Analysis of the pACS sequence revealed the presence of two putative MXREs (MXRE1 and MXRE2) at −780 and −742 bp, which were designated MXRE1 and MXRE2, respectively (Fig. 4C). Point mutations that abolish Mxr1p binding to pAOXI-MXREs (2) were introduced into either (M1 and M2) or both (M3)

![Figure 3](https://example.com/figure3.png)

**FIGURE 3. Regulation of ACS1 and AOX1 expression by Mxr1pN400 and Mxr1pS215A.** A, schematic of the N-terminal region of Mxr1p. Major functional domains are indicated. Serine 215, whose phosphorylation is essential for interaction with 14-3-3 protein, is indicated by an asterisk. B, analysis of Mxr1p expression by qPCR in different P. pastoris strains as indicated. C, analysis of the expression of Mxr1p and Mxr1pN400 by Western blotting using anti-c-myc antibodies in the Pp-Mxr1-OE and Pp-Mxr1N400-OE strains, respectively. PGK served as a loading control. D, analysis of ACS1 expression by qPCR in different P. pastoris strains cultured in YPA. E, Western blot analysis of ACS1mRNA levels in different P. pastoris strains cultured in YPA. PGK was used as a loading control. F, quantification of the data in E. The intensity of individual bands was quantified and expressed as arbitrary units ± S.D. relative to controls. Data are the average of three independent experiments. G, growth curves of different P. pastoris strains cultured in YPA medium. H, analysis of the expression of Mxr1p and Mxr1pS215A by Western blotting using anti-c-myc antibodies in the Pp-Mxr1-OE and Pp-Mxr1S215A-OE strains, respectively. PGK served as a loading control. I, analysis of ACS1 expression by qPCR in different P. pastoris strains cultured in YPA. J, Western blot analysis of ACS1mRNA levels in different P. pastoris strains cultured in YPA. PGK was used as a loading control. K, quantification of the data in J. The intensity of individual bands was quantified and expressed as arbitrary units ± S.D. relative to controls. Data are the average of three independent experiments. L, analysis of AOX1 expression by qPCR in different P. pastoris strains in YPA. M, analysis of AOX1 expression by qPCR in different P. pastoris strains cultured in YMP. Error bars indicate mean ± S.D. *, p < 0.05; **, p < 0.005; ***, p < 0.0005; ns, not significant. One-way analysis of variance followed by Tukey’s multiple comparison test was done (n = 3).
MXREs of pACS1 (Fig. 4D), and reporter gene expression was examined. The results indicate that mutations in either or both MXREs abrogate Mxr1p-dependent activation of pACS1 (Fig. 4E). Furthermore, insertion of a 10- or 20-bp sequence between the two MXREs abrogates Mxr1p-dependent activation of pACS1 (Fig. 4, F and G). Mxr1p-dependent activation of ACS1 expression is more efficient in cells cultured in YPA than in those cultured in YNBA + CAA or YNBA + Glu (Fig. 4H). Therefore, in addition to acetate and amino acids, other components present in YPA also contribute to the transcriptional activation of ACS1 by Mxr1p.

To demonstrate Mxr1p binding to pACS-MXREs, oligonucleotides carrying mutations in either or both the MXREs were synthesized (Fig. 5A) and radiolabeled, and their ability to bind to recombinant Mxr1pN150 and Mxr1pN400 encoding 150 and 400 N-terminal amino acids, respectively (Fig. 5, B and C), was examined in an EMSA. Two DNA-protein complexes (I and II) were generated when these proteins were incubated with the pACS1-WT probe (Fig. 5D). Only complex I was formed with pACS1-M1 and M2 probes. Protein-DNA complex formation was abrogated when incubated with pACS1-M3 (Fig. 5D). Addition of anti-His or anti-GST antibodies resulted in either a supershift or abrogation of DNA-protein complexes, confirming the presence of recombinant Mxr1pN150 or Mxr1pN400 in these complexes, respectively (Fig. 5, E and F).

Discussion

In this study, we demonstrate that Mxr1p, a transcriptional activator of genes of the mut pathway, is a key regulator of the acetate utilization pathway in P. pastoris. Mxr1p activates the expression of ACS1 by binding to two MXREs present in pACS1. Mutations in either of the pACS1-MXREs and a 10- or 20-bp increase in the distance between the two MXREs abrogate trans-activation by Mxr1p. Therefore, occupation of both MXREs by Mxr1p and close proximity (30 bp) between two MXREs is essential for trans-activation of pACS1 by Mxr1p. Therefore, two MXREs separated by a 30-bp spacer function as an MxRU of pACS1. Although recombinant Mxr1pN150 and Mxr1pN400 bind to both pACS1-M1 and M2 probes in vitro, formation of both complex I or II is essential for trans-activation by full-length Mxr1p in vivo. It will be interesting to examine the binding of full-length Mxr1p to pACS1-MXREs in vitro. So far, we have not been successful in generating recombinant full-length Mxr1p, and DNA binding studies with P. pastoris

**FIGURE 4. Study of lacZ expression from pACS and identification of pACS-MXREs.** A, schematic of pACS-lacZ constructs. B, estimation of β-galactosidase activity in lysates of cells transformed with pACS-lacZ constructs. Cells were cultured in YPA or YNBA. C, nucleotide sequence of pACS between −809 and −737 bp. MXRE1 and MXRE2 are underlined. The cytosine residue within the MXRE crucial for Mxr1p binding is indicated by an asterisk. D, schematic of pACS1-lacZ constructs containing wild-type and mutant MXREs. Point mutations within MXREs are underlined. E, estimation of β-galactosidase activity in lysates of cells transformed with pACS-lacZ constructs. Cells were cultured in YPA. F, schematic of pACS-lacZ constructs carrying 10- or 20-bp insertions between the two MXREs. F, schematic of pACS1-lacZ constructs carrying 10- or 20-bp insertions between the two MXREs. G, effect of insertion of 10- or 20-bp insertions between the two MXREs on β-galactosidase activity in lysates of cells transformed with pACS1-lacZ constructs. β-Galactosidase activity measurements represent the mean ± S.D. of data from three independent experiments. H, lacZ expression from pACS1 in cells cultured in various media as indicated.
cell extracts have not been conclusive. Taken together, the results of this study indicate that Mxr1p binding to two pACS1-MXREs separated by a 30-bp spacer is essential for trans-activation from pACS1, as shown schematically in Fig. 6. The inability of Adr1p to restore ACS1 expression and growth in /H9004 mxr1 indicates that Adr1p is not a functional homologue of Mxr1p. This is consistent with our earlier observation where we demonstrated that the DNA binding specificity of Mxr1p is different from that of Adr1p (2). Mxr1p overexpression studies clearly demonstrate that the growth rate of P. pastoris in YPA medium is directly proportional to the level of expression of Mxr1p and ACS1. Furthermore, the trans-activation domain in the N-terminal region has an important role in

FIGURE 5. Analysis of binding of recombinant Mxr1pN150 and Mxr1pN400 to pACS-MXREs by EMSA. A, nucleotide sequence of oligonucleotides containing wild-type and mutant MXREs. MXREs are boxed. B, schematic of histidine-tagged Mxr1pN150 and GST-tagged Mxr1pN400. C, SDS-PAGE profile of Mxr1pN150 and Mxr1pN400 purified from E. coli lysates by nickel-nitrilotriacetic acid and glutathione affinity chromatography, respectively. D, analysis of binding of Mxr1pN150 and Mxr1pN400 to 32P-labeled oligonucleotides containing wild-type and mutant MXREs by EMSA. Complexes I and II are generated when Mxr1pN150 and Mxr1pN400 bind to both MXREs. Only complex I is obtained when Mxr1pN150 and Mxr1pN400 bind to only one of the MXREs. E, supershift of complexes I and II formed by His-tagged Mxr1pN150 by the addition of anti-His antibodies. F, abrogation of the formation of complexes I and II by GST-tagged Mxr1pN400 by addition of anti-GST antibodies.

FIGURE 6. Schematic of Mxr1p-mediated trans-activation of pACS1 in P. pastoris cells cultured in YPA. A point mutation in MXRE1 or MXRE2 abrogates Mxr1p binding to pACS1 in vitro and trans-activation of pACS1-lacZ in vivo. An increase in the distance between the two MXREs also abolishes trans-activation in vivo. Therefore, the two MXREs separated by a 30-bp region functions as an MxRU.
the regulation of ACS1 expression and growth, as evident from the ability of Mxr1p \(^{N400}\) to restore ACS1 expression and growth of \(\Delta mxr1\). The differences in ACS1 expression levels and growth rates of \(Pp-Mxr1-OE\) and \(Pp-Mxr1^{N400,OE}\) indicate that another trans-activation domain, present between amino acids 401 and 1155, also has a key role in the activation of ACS1 expression. Studies with \(Pp-Mxr1^{S215A,OE}\) indicate that the serine 215 residue of Mxr1p has no role in the regulation of ACS1 expression.

Mxr1p localizes to the nuclei of cells cultured in several non-fermentable carbon sources. However, its target genes in the mut pathway are activated only in selected non-fermentable carbon sources such as methanol. Repression of Mxr1p target genes in other non-fermentable carbon sources such as ethanol is facilitated by its interaction with 14-3-3 protein (9). Phosphorylation of serine 215 of Mxr1p is crucial for this interaction, and S215A mutation abrogates the Mxr1p/14-3-3 protein interaction (9). Having identified ACS1 as a novel target gene of Mxr1p, it was of interest to examine the role of the S215A mutation on ACS1 expression. The results indicate that the S215A mutation does not affect Mxr1p-mediated trans-activation of ACS1 in cells cultured in YPA.

The differential localization of Mxr1p in cells cultured in YNBA, YNBA + Glu, YNBA + CAA, and YPA led us to investigate the role of amino acids in the regulation of ACS1 expression by Mxr1p. Although Mxr1p localizes to the nucleus of cells cultured in YNBA + Glu, YNBA + CAA, and YPA, Mxr1p-dependent growth was observed only in cells cultured in YNBA + CAA and YPA. Furthermore, Mxr1p-dependent lacZ expression from the ACS1 promoter is more efficient in cells cultured in YPA than in those cultured in YNBA + CAA. Therefore, in addition to amino acids, other components in YPA medium may contribute to the regulation of ACS1 expression by Mxr1p. The fact that acetate, together with glutamate or casamino acids, triggers the nuclear translocation of Mxr1p opens up new avenues of study of the mechanism of nuclear translocation of Mxr1p.

The differential regulation of acetate metabolism by Mxr1p in minimal and nutrient-rich media is similar to that of the Rop1p-mediated regulation of methanol metabolism. Rop1p translocates to the nucleus in cells cultured in YPM and represses transcription of genes of the mut pathway by competing with Mxr1p for binding to MXREs. However, Rop1p is cytosolic in cells cultured in YNBM and has no role in the regulation of methanol metabolism. The physiological significance of differential regulation of methanol metabolism by Rop1p can be explained as follows. Unlike S. cerevisiae, P. pastoris can utilize amino acids as a source of carbon. In cells cultured in YPM, utilization of amino acids is preferred to that of methanol, and, therefore, to minimize methanol utilization, Rop1p translocates to the nucleus and represses the expression of genes of the mut pathway. In YNBM, methanol is the sole source of carbon, and, therefore, Rop1p remains in the cytosol, facilitating the efficient activation of genes of the mut pathway by Mxr1p. Therefore, Mxr1p and Rop1p function as nutrient sensors and differentially regulate acetate and methanol metabolism, respectively, in cells cultured under minimal and nutrient-rich conditions.

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