THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 291, NO. 39, pp. 20588–20601, September 23, 2016
© 2016 by The American Society for Biochemistry and Molecular Biology, Inc. Published in the U.S.A.

Methanol Expression Regulator 1 (Mxr1p) Is Essential for the Utilization of Amino Acids as the Sole Source of Carbon by the Methylotrophic Yeast, *Pichia pastoris*[*][S]

Received for publication, May 25, 2016, and in revised form, July 26, 2016 Published, JBC Papers in Press, August 12, 2016, DOI 10.1074/jbc.M116.740191

Umakant Sahu and Pundi N. Rangarajan[1]
From the Department of Biochemistry, Indian Institute of Science, Bangalore 560012, India

Unlike *Saccharomyces cerevisiae*, the methylotrophic yeast *Pichia pastoris* can assimilate amino acids as the sole source of carbon and nitrogen. It can grow in media containing yeast extract and peptone (YP), yeast nitrogen base (YNB) + glutamate (YNB + Glu), or YNB + aspartate (YNB + Asp). Methanol expression regulator 1 (Mxr1p), a zinc finger transcription factor, is essential for growth in these media. Mxr1p regulates the expression of several genes involved in the utilization of amino acids as the sole source of carbon and nitrogen. These include the following: (i) *GDH2* encoding NAD-dependent glutamate dehydrogenase; (ii) *AAT1* and *AAT2* encoding mitochondrial and cytosolic aspartate aminotransferases, respectively; (iii) *MDH1* and *MDH2* encoding mitochondrial and cytosolic malate dehydrogenases, respectively; and (iv) *GLN1* encoding glutamine synthetase. Synthesis of all these enzymes is regulated by Mxr1p at the level of transcription except *GDH2*, whose synthesis is regulated at the level of translation. Mxr1p activates the transcription of *AAT1, AAT2,* and *GLN1* in cells cultured in YP as well as in YNB + Glu media, whereas transcription of *MDH1* and *MDH2* is activated in cells cultured in YNB + Glu but not in YP. A truncated Mxr1p composed of 400 N-terminal amino acids activates transcription of target genes in cells cultured in YP but not in YNB + Glu. Mxr1p binds to Mxr1p response elements present in the promoters of *AAT2, MDH2,* and *GLN1*. We conclude that Mxr1p is essential for utilization of amino acids as the sole source of carbon and nitrogen, and it is a global regulator of multiple metabolic pathways in *P. pastoris*.

Saccharomyces cerevisiae can utilize NH_4^+ or amino acids such as L-glutamate as the source of nitrogen but not as the sole source of carbon and energy. When *S. cerevisiae* is cultured in media containing NH_4^+ as the source of nitrogen, glutamate dehydrogenase encoded by *GDH1* or *GDH3* functions as an anabolic enzyme catalyzing the reaction between NH_4^+ and α-ketoglutarate to generate glutamate with NADPH acting as a co-factor (1). When cultured in media containing glutamate as the sole source of nitrogen, glutamate dehydrogenase encoded by *GDH2* acts as a catabolic enzyme by converting glutamate to α-ketoglutarate and NH_4^+ in the presence of NADH. The NH_4^+ thus generated is used for the biosynthesis of glutamine (2). Unlike *S. cerevisiae*, certain yeasts can utilize L-amino acids such as glutamate, aspartate, and proline as the sole source of carbon as well as nitrogen. These include the following: *Scheffersomyces stipitis* (also known as *Pichia stipitis*), *Candida albicans, Candida maltosa, Candida shehatae, Candida glabrata, Candida reukaufii, Candida utilis, Debaryomyces hansenii, Kluyveromyces lactis, Kluyveromyces marxianus, Lodderomyces elongisporus, Meyerozyma guilliermondii, Pichia capsulata, Yarrowia lipolytica, Rhodotorula rubra*, and *Trichosporon beigelii* (3). The assimilation of glutamate requires the activity of NAD-dependent glutamate dehydrogenase 2 (referred to as GDH2 in this study), and a Δ*gdh2* strain cannot utilize glutamate or aspartate as either a carbon or a nitrogen source as demonstrated in the case of *S. stipitis* (3).

In addition to GDH2, enzymes such as aspartate aminotransferase (AAT),[2] malate dehydrogenase (MDH), and glutamine synthetase (GLN1) also play key roles in the metabolism of amino acids (4). In *S. cerevisiae*, *AAT1* and *AAT2* encode AAT localized in mitochondria (mAAT) and cytoplasm (cAAT). cAAT catalyzes the reversible conversion of glutamate and oxaloacetate into α-ketoglutarate and aspartate (4). The oxaloacetate thus generated is converted to malate by MDH present in the cytoplasm (cMDH) encoded by *MDH2*. Malate enters mitochondria via α-ketoglutarate-malate exchanger protein, which also transports α-ketoglutarate in the opposite direction. Malate is oxidized to oxaloacetate by the mitochondrial MDH (mMDH) encoded by *MDH1*, resulting in the formation of NADH, which enters the electron transport chain and generates energy (4). The mitochondrial oxaloacetate is converted to aspartate by mAAT, which is transported to cytoplasm via the aspartate-glutamate exchanger. Glutamate present in the cytoplasm is converted to glutamine by GLN1 (4).

P. pastoris, a methylotrophic yeast, is extensively used for the production of recombinant proteins. Being a respiratory yeast, *P. pastoris* completely oxidizes sugars, avoiding formation of ethanol, and this results in efficient utilization of carbon sources yielding high biomass. During high cell density fermentation of *P. pastoris*, ammonium sulfate or organic nitrogen sources such as amino acids are used as nitrogen sources. Sev-

* This work was supported by Research Grant BT/PR3889/BRB/10/996/2011 from the Department of Biotechnology, New Delhi, and J. C. Bose Fellowship Grant SB/S2/JCB-025/2015 by the Science and Engineering Research Board, New Delhi, India (to P. N. R.). The authors declare that they have no conflicts of interest with the contents of this article.
[S] This article contains supplemental data.
[1] To whom correspondence should be addressed: Dept. of Biochemistry, FE15, Biology Bldg., Indian Institute of Science, Bangalore 560012, India. Tel./Fax: 918023601492; E-mail: pnr@biochem.iisc.ernet.in.
This is an Open Access article under the CC BY license.

[2] The abbreviations used are: AAT, aspartate aminotransferase; YNB, yeast nitrogen base; YP, yeast extract and peptone; m, mitochondria; c, cytoplasm; MDH, malate dehydrogenase; qPCR, quantitative PCR.

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

Strain	Genotype	Ref.
GS115	*his4*	5
Δ*mxr1*	*GS115, Ppmxr1*Δ::*Zeocin*^{r}	13
Δ*prm1*	*GS115, Pprm1*Δ::*Zeocin*^{r}	10
Δ*rop1*	*GS115, Pprop1*Δ::*Zeocin*^{r}	9
Δ*gdh2*	*GS115, Ppgdh2*Δ::*Zeocin*^{r}	This study
Δ*aat1*	*GS115, Ppaat1*Δ::*Zeocin*^{r}	This study
Δ*aat2*	*GS115, Ppaat2*Δ::*Zeocin*^{r}	This study
Δ*mdh1*	*GS115, Ppmdh1*Δ::*Zeocin*^{r}	This study
Δ*mdh2*	*GS115, Ppmdh2*Δ::*Zeocin*^{r}	This study
Δ*gdh2-GDH2*^{His}	Δ*gdh2, his4*:: (P_{GDH2}*PpGDH2-His*)	This study
GS115-GDH2^{His}	*GS115, his4*:: (P_{GDH2}*PpGDH2-His*)	This study
Δ*mxr1-GDH2*^{His}	Δ*mxr1, his4*:: (P_{GDH2}*PpGDH2-His*)	This study
MXR1^{Myc}-OE	Δ*mxr1, Blasticidin*^{r}:: (P_{GAPDH}*PpMXR1-Myc*)	13
MXR1^{Myc}*-OE-GDH2*^{His}	*MXR1*^{Myc}*-OE, his4*:: (P_{GDH2}*PpGDH2-His*)	This study
MXR1^{N400}*-OE*	Δ*mxr1, Blasticidin*^{r}:: (P_{GAPDH}*PpMXR1*^{N400}*-Myc*)	13
MXR1^{N400}*-OE-GDH2*^{His}	*MXR1*^{N400}*-OE, his4*:: (P_{GDH2}*PpGDH2-His*)	This study
Δ*mxr1-GDH2-OE1*	Δ*mxr1, Zeocin*^{r}:: (P_{GAPDH}*PpGDH2-Myc*)	This study
Δ*mxr1-GDH2-OE2*	Δ*mxr1, Zeocin*^{r}:: (P_{CUP}*PpGDH2-Myc*)	This study
AAT1^{Myc}	*GS115, Zeocin*^{r}:: (P_{AAT1}*PpAAT1-Myc*)	This study
AAT2^{Myc}	*GS115, Zeocin*^{r}:: (P_{AAT2}*PpAAT2-Myc*)	This study
MDH1^{His}	*GS115, his4*:: (P_{MDH1}*PpGDH2-His*)	This study
MDH2^{His}	*GS115, his4*:: (P_{MDH2}*PpMDH2-His*)	This study
GLN1^{Myc}	*GS115, Zeocin*^{r}:: (P_{GLN1}*PpGLN1-Myc*)	This study

eral transcription factors that regulate carbon metabolism have been identified in *P. pastoris.* These include the following: Mxr1p, Rop1p, Trm1p, Mit1p, and Nrg1p (5–14). The role of these transcription factors in the regulation of amino acid metabolism has not been examined. Here, we demonstrate that *P. pastoris* can utilize amino acids as the sole source of carbon, and Mxr1p but not Trm1p or Rop1p is essential for this process. Mxr1p regulates the synthesis of several key enzymes involved in amino acid metabolism such as GDH2, AAT1, AAT2, MDH1, MDH2, and GLN1 at the transcriptional or post-transcriptional level.

Results

Enzymes Essential for the Utilization of Amino Acids as the Sole Source of Carbon by P. pastoris and Regulation of Their Biosynthesis by Mxr1p—The ability *of P. pastoris* to utilize amino acids as the sole source of carbon and nitrogen has not been investigated. We therefore examined the ability of *P. pastoris GS115* strain to grow in media such as yeast nitrogen base (YNB) without amino acids and 0.5% ammonium sulfate supplemented with 2.0% glucose (YNBD), 1.0% glutamate (YNB + Glu), 1% aspartate (YNB + Asp) or YNB + Glu without ammonium sulfate. The results indicate that *P. pastoris GS115* strain but not Δ*mxr1* strain (Table 1) can grow in these media (Fig. 1*A*) indicating that Mxr1p is essential for the utilization of amino acids as the sole source of carbon and nitrogen. Rop1 and Trm1p, which regulate the expression of genes of methanol utilization pathway (8, 9), have no role in the utilization of amino acids because growth of Δ*rop1* and Δ*trm1* strains is not affected when cultured in YP medium (1.0% yeast extract and 2.0% peptone) (Fig. 1*A*). *S. cerevisiae* was unable to grow in YP medium as expected (Fig. 1*B*) (3). Subcellular localization studies employing *P. pastoris* strain expressing a FLAG-tagged Mxr1p (13) indicates that Mxr1p localizes to the nucleus of cells cultured in YP as well as YPM (YP + 2% methanol) but was cytosolic in cells cultured in YPD (YP + 2% glucose) (Fig. 1*C*). Cell lysates of *GS115* and Δ*mxr1* strains cultured in YP were subjected to SDS-PAGE, and proteins were visualized by Coomassie Blue staining (Fig. 1*D*). Proteins a–f, which are differentially expressed in *GS115* and Δ*mxr1,* were selected; protein bands were excised and subjected to in-gel trypsin digestion, and the tryptic peptides were analyzed by MALDI-TOF mass spectrometry (supplemental data). Proteins a– c were identified as GDH2, alcohol oxidase, and formate dehydrogenase, respectively, and proteins d–f were identified as aconitase, malate synthase, and citrate synthase, respectively (Fig. 1*D* and supplemental data). To confirm their differential expression in *GS115* and Δ*mxr1,* qPCR analysis was carried out with RNA isolated from cells cultured in YP medium. The results indicate that *AOXI* and *FDH* transcripts are present in higher levels in *GS115* than Δ*mxr1,* whereas transcript levels of *ACO*, *MS,* and *CS* are higher in Δ*mxr1* than *GS115* cultured in YP (Fig. 1*E*). However, *GDH2* mRNA levels were comparable in *GS115* and Δ*mxr1* cultured in YP (Fig. 1*E*). Because GDH2 has a key role in the utilization of amino acids in *S. stipitis* and GDH2 protein but not mRNA levels were consistently lower in Δ*mxr1* than *GS115* in several independent experiments, a detailed study was undertaken. *P. pastoris GS115* and Δ*mxr1* were transformed with *pIB3-GDH2*^{His}, and expression of Gdh2p^{His} was examined in cells cultured in different carbon sources by Western blotting. GDH2^{His} expression levels were higher in cells cultured in YP, YNB + Glu, and YNB + Asp than those cultured in YPD or YNBD (Fig. 1, *F* and *G*). Furthermore, GDH2^{His} is expressed at higher levels in *GS115* than Δ*mxr1* cultured in YP, YNB + Glu, and YNB + Asp media (Fig. 1, *F* and *G*). Immunofluorescence studies indicate that GDH2^{His} is localized to the cytosol (Fig. 1*H*). Thus, Mxr1p is required for the synthesis of Gdh2p but not *GDH2* mRNA suggesting that Mxr1p regulates *GDH2* expression at the post-transcriptional level.

To confirm whether GDH2 is required for the utilization of amino acids as the sole source of carbon, *P. pastoris* Δ*gdh2* strain was generated (Fig. 2, *A* and *B*), and its ability to grow in YPD, YP, YNB + Glu, and YNB + Asp media was examined. Growth of Δ*gdh2* strain was normal in YPD medium but severely impaired when cultured in YP, YNB + Glu, and YNB +

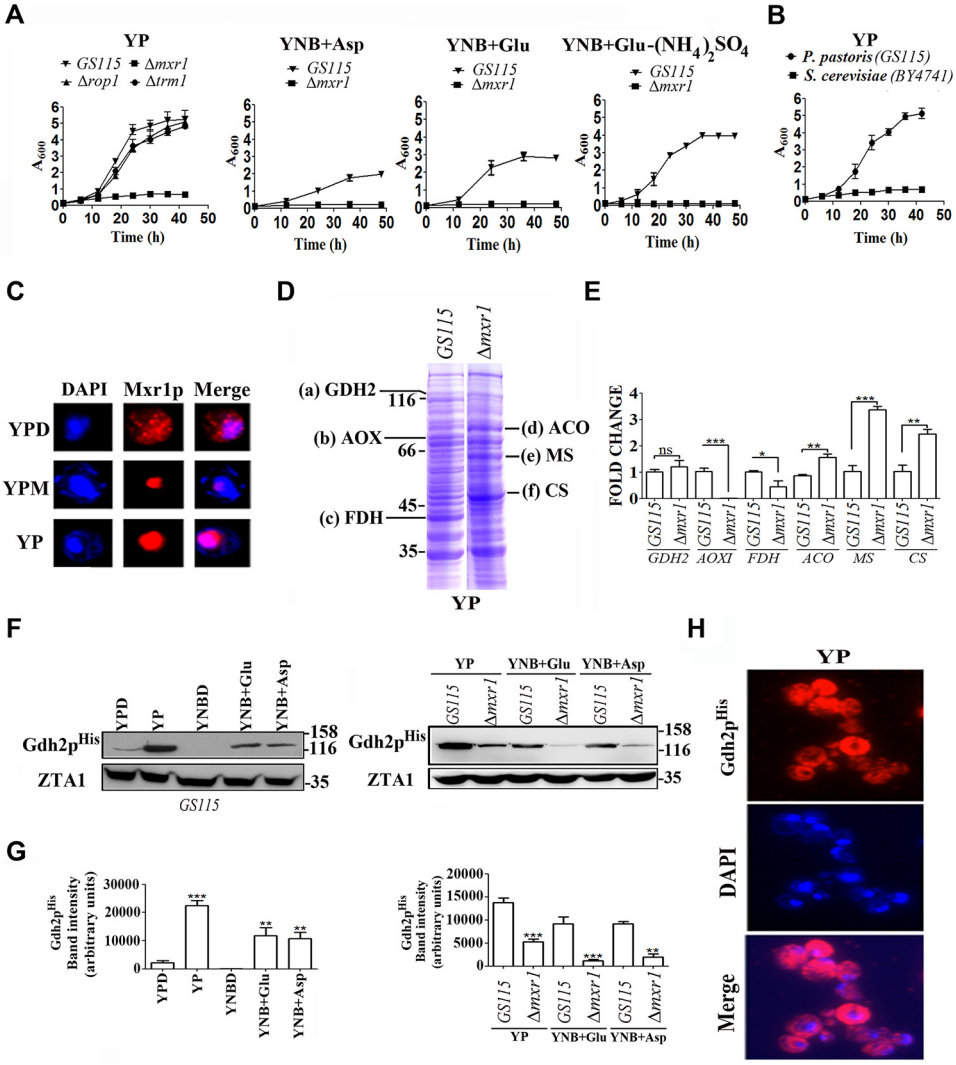


FIGURE 1. **Growth of *P. pastoris GS115* and Δ*mxr1* in media containing amino acids as the sole source of carbon and regulation of GDH2 expression by Mxr1p.** *A*, growth of *P. pastoris GS115* and Δ*mxr1* strains in media containing amino acids as the sole source of carbon and nitrogen. In YNB + Glu-$(NH_4)_2SO_4$ medium, glutamate serves as the sole source of carbon as well as nitrogen. *B*, comparison of growth of *P. pastoris* and *S. cerevisiae* in YP medium. *C*, subcellular localization of FLAG-tagged Mxr1p in cells cultured in different media as analyzed by immunofluorescence using anti-FLAG antibodies. DAPI was used to stain the nucleus. *D*, protein profile of lysates of cells cultured in YP medium. Proteins were resolved on SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R. Proteins a–f, which are differentially expressed in *GS115* and Δ*mxr1*, are identified by mass spectrometry (see supplemental data). *E*, qPCR validation of genes differentially expressed in *GS115* and Δ*mxr1*. *F*, Western blotting analysis of lysates of *GS115-GDH2*^{His} and Δ*mxr1-GDH2*^{His} strains expressing Gdh2p^{His} using anti-His tag antibodies. Cells were cultured in different media as indicated. ZTA1 (23) served as loading control. *G*, quantitation of data presented in *F*. *H*, cytosolic localization of Gdh2p^{His} in cells cultured in YP as examined by immunofluorescence using anti-His tag antibodies. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$; *ns*, not significant.

Asp media (Fig. 2*C*). *pGAPBA-MXR1* was constructed and transformed into Δ*mxr1* to generate *MXR1*^{Myc}*-OE* strain. Similarly, *pIB3-GDH2*^{His} was constructed and transformed into Δ*gdh2* to generate Δ*gdh2-GDH2*^{His} strain. Expression of Mxr1p^{Myc} and Gdh2p^{His} was confirmed by Western blotting using anti-Myc and anti-His tag antibodies, respectively (Fig. 2, *D* and *E*). Growth of *MXR1*^{Myc}*-OE* and Δ*gdh2-GDH2*^{His} strains in YP, YNB + Glu, and YNB + Asp media was comparable with that of *GS115* (Fig. 2*F*) confirming that Mxr1p and GDH2 are essential for the utilization of amino acids as the sole source of carbon.

Differential Regulation of GDH2 Expression by MXR1 and MXR1^{N400}—The first 400 amino acids of Mxr1p (Mxr1p^{N400}) contain 14-3-3 protein interaction region between amino acids 212 and 225 and a trans-activation domain between amino acids 246 and 280 (Fig. 3*A*) (13, 14). This trans-activation domain mediates carbon source-dependent repression and activation of Mxr1p-regulated genes (13, 14). To examine the role of this N-terminal trans-activation domain in the regulation of *GDH2* expression, *P. pastoris* Δ*mxr1* strain overexpressing Mxr1p^{N400} was generated and named *MXR1*^{N400}*-OE* (Fig. 3*A*). Expression of *MXR1* and *MXR1*^{N400} mRNA in cells cultured in YP and YNB + Glu media was confirmed by qPCR (Fig. 3*B*). Overexpression of Mxr1p^{N400} resulted in partial restoration of growth of cells cultured in YP but not YNB + Glu (Fig. 3*C*). Overexpression of Mxr1p but not Mxr1p^{N400} results in the restoration of Gdh2p^{His} levels in Δ*mxr1* (Fig. 3, *D* and *E*). However, deletion or overexpression of Mxr1p or Mxr1p^{N400} had no significant effect on *GDH2* mRNA levels (Fig. 3*F*). These results not only confirm post-transcriptional regulation of *GDH2*

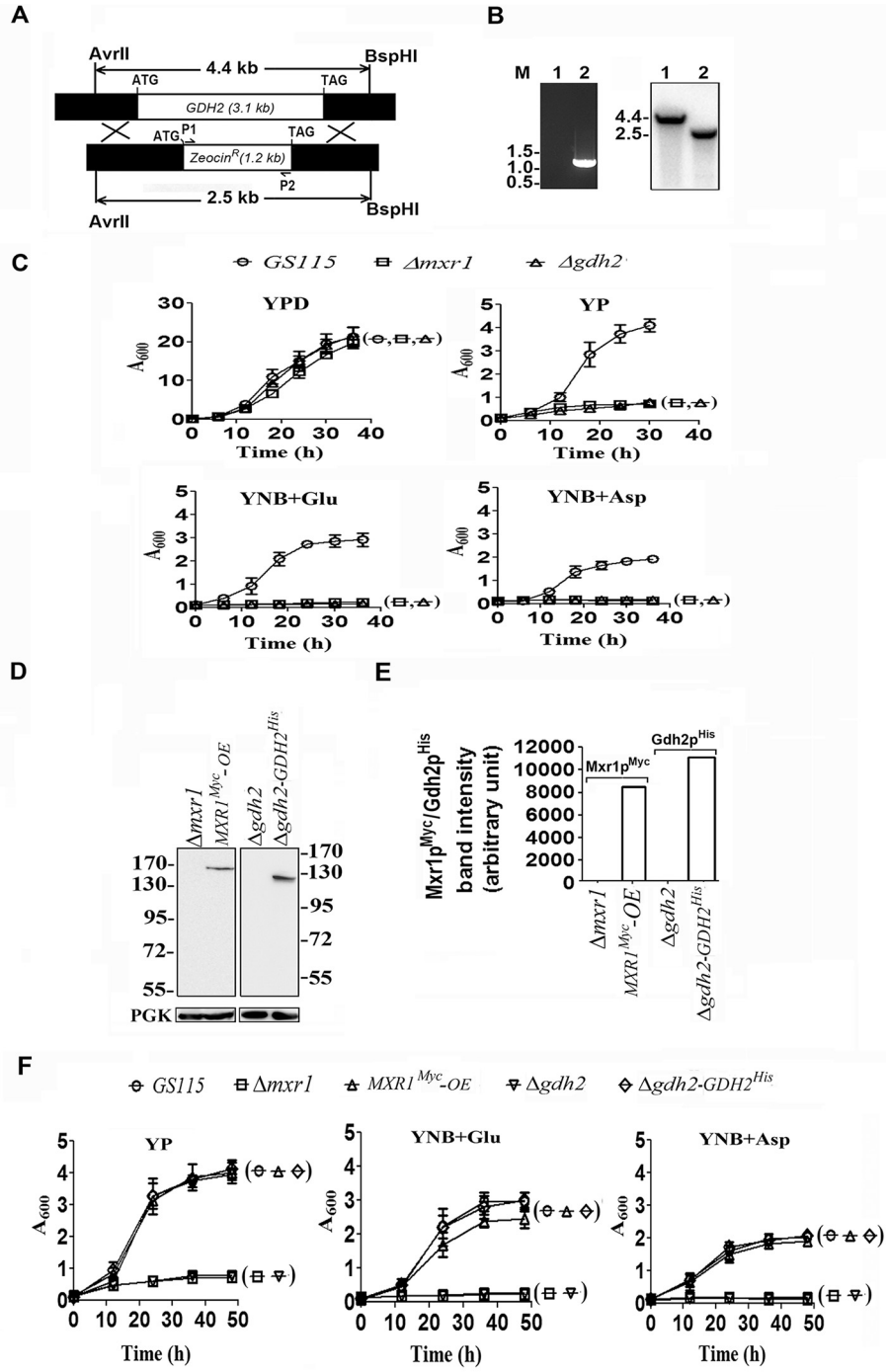


FIGURE 2. **Analysis of the role of GDH2 in the utilization of amino acids as the sole source of carbon.** *A,* strategy for the generation of Δ*gdh2* strain. The restriction enzymes used for the digestion of genomic DNA for use in Southern analysis are indicated. P1, P2 primer pairs used in PCRs are indicated. *B,* PCR and Southern blotting analysis of genomic DNA isolated from *GS115* (*1*) and Δ*gdh2* (*2*) strains. The Zeocin expression cassette is amplified by PCR only in Δ*gdh2* strain. *M*, DNA molecular weight markers (kb). The 4.4- and 2.5-kb bands hybridize to a radiolabeled probe (−571 to −24 bp of *GDH2*) in the Southern blot as expected. *C,* analysis of growth of *GS115*, Δ*mxr1*, and Δ*gdh2* strains cultured in media containing different carbon sources. *D,* Western blotting analysis of Δ*mxr1* overexpressing Mxr1p from *GAPDH* promoter as Myc-tagged protein and Δ*gdh2* expressing Gdh2p from its own promoter as His-tagged protein. Anti-Myc and anti-His tag antibodies were used to detect Mxr1p and Gdh2p, respectively. *E,* quantitation of data presented in *D*. *F,* analysis of growth of different *P. pastoris* strains in various media as indicated.

expression by Mxr1p but also demonstrate that the N-terminal 400 amino acids alone are not sufficient for this process.

Mxr1p Is a Key Regulator of Aspartate Aminotransferase—Because the growth of Δ*gdh2* can be restored by the expression of GDH2, it was of interest to examine whether overexpression of GDH2 in Δ*mxr1* can restore the growth in YP and YNB + Glu media. GDH2 was expressed as a Myc-tagged protein ($Gdh2p^{Myc}$) at high levels from the *GAPDH* promoter or moderate levels from *S. cerevisiae CUP1* promoter (15) in Δ*mxr1* to generate Δ*mxr1-GDH2-OE1* and Δ*mxr1-GDH2-OE2* strains respectively. $Gdh2p^{Myc}$ expression in cells cultured in YP medium was confirmed by Western blotting with anti-Myc tag

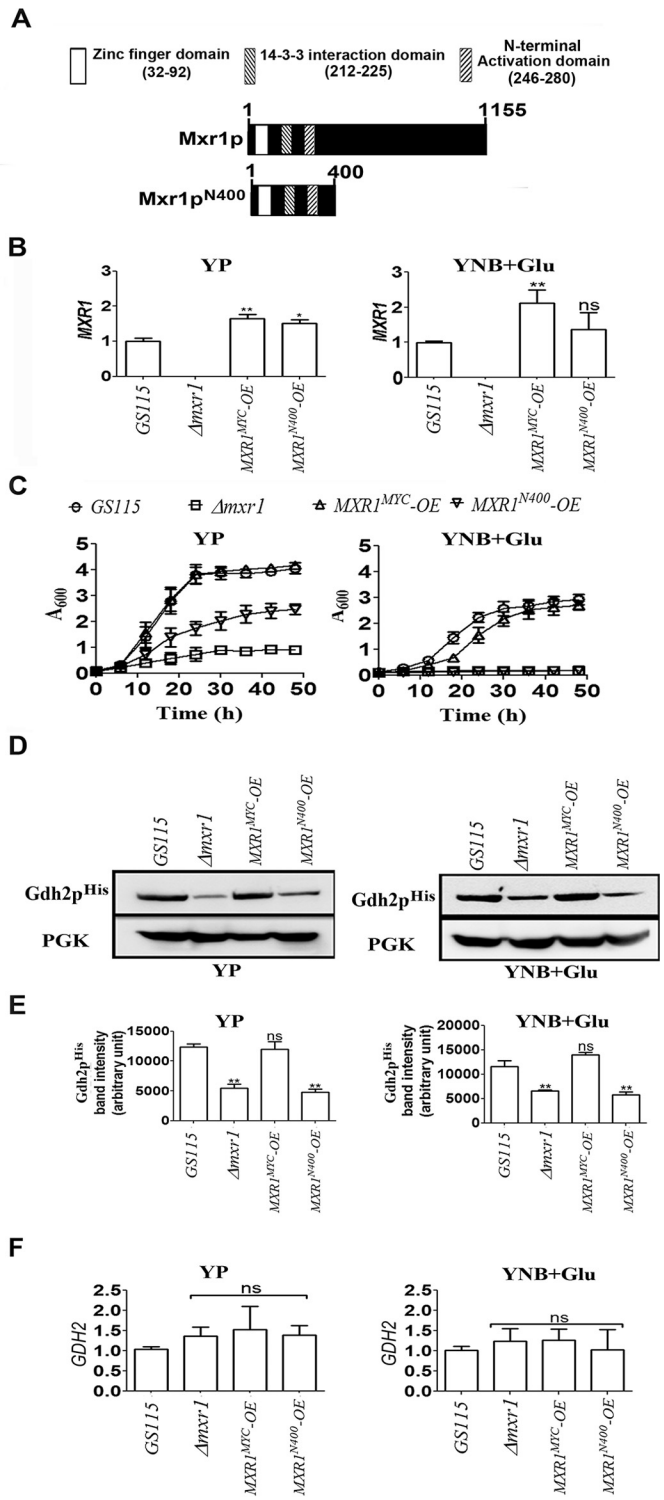


FIGURE 3. **Regulation of *GDH2* expression by Mxr1p and Mxr1p^{N400}.** *A,* schematic representation of Mxr1p and Mxr1p^{N400}. The major domains within the 400 N-terminal amino acids are indicated. *B, MXR1* and *MXR^{N400}* mRNA in different *P. pastoris* strains cultured in YP and YNB + Glu media as analyzed by qPCR. *C,* effect of overexpression of Mxr1p and Mxr1p^{N400} on the growth of Δ*mxr1* cultured in YP and YNB + Glu media. *D,* Western blotting analysis of Gdh2p^{His} levels in Δ*mxr1* overexpressing Mxr1p and Mxr1p^{N400}. The gene encoding Gdh2p^{His} was expressed from its own promoter in different strains as indicated. *E,* quantitation of data presented in *D*. *F,* analysis of *GDH2* mRNA levels by qPCR in different *P. pastoris* strains cultured in YP and YNB + Glu media. *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 carried out ($n = 3$).

antibodies (Fig. 4, *A* and *B*). Overexpression of Gdh2p^{Myc} from either of these promoters was unable to restore growth of Δ*mxr1* in YP and YNB + Glu media (Fig. 4*C*). The fact that expression of GDH2 results in the restoration of growth of Δ*gdh2* (Fig. 2*F*) but not Δ*mxr1* strain indicates that Mxr1p may regulate the expression of other enzymes that are required for utilization of amino acids as the sole source of carbon in *P. pastoris*.

To examine whether mAAT and cAAT are required for the utilization of amino acids as the sole source of carbon in *P. pastoris,* they were expressed as Myc-tagged proteins, and their localization in mitochondria and cytoplasm, respectively, was confirmed by immunofluorescence using anti-Myc tag antibodies (Fig. 5*A*). *P. pastoris* Δ*aat1* and Δ*aat2* strains were generated, and their growth was examined in media containing different carbon sources. The results indicate that Δ*aat2* strain is unable to grow in YP, YNB + Glu, and YNB + Asp media indicating that cAAT is essential for the growth of *P. pastoris* in these media (Fig. 5*B*). qPCR analysis indicates that *AAT1* and *AAT2* are expressed at low levels in Δ*mxr1* strain (Fig. 5*C*). Overexpression of either Mxr1p or Mxr1p-^{N400} in Δ*mxr1* strain results in up-regulation of *AAT1* and *AAT2* expression in cells cultured in YP medium. However, only Mxr1p but not Mxr1p^{N400} restores the expression of these genes in cells cultured in YNB + Glu (Fig. 5*C*).

Mxr1p Regulates the Expression of Genes Encoding mMDH, cMDH, and GLN1—In view of the crucial role of mMDH and cMDH in the transport of malate between cytoplasm and mitochondria (4), a detailed study was undertaken to examine their role during the utilization of amino acids as the sole source of carbon in *P. pastoris*. Immunofluorescence studies indicate that mMDH and cMDH are indeed localized in mitochondria and cytoplasm, respectively, in *P. pastoris* cultured in YP medium (Fig. 6*A*). *P. pastoris* Δ*mdh1* and Δ*mdh2* strains were generated, and their growth was examined in different media. The results indicate that the growth of Δ*mdh1* is severely impaired in cells cultured in media containing glucose or amino acids as the sole source of carbon (Fig. 6*B*). However, the growth of Δ*mdh2* is affected only in cells cultured in YP or YNB + Glu media (Fig. 6*B*). Thus, the generation of malate in the cytoplasm and its conversion to oxaloacetate in the mitochondria are important for the normal growth of cells cultured in YP and YNB + Glu media. The expression of *MDH1* as well as *MDH2* is regulated by Mxr1p only in cells cultured in YNB + Glu but not YP medium as evident from qPCR studies (Fig. 6*C*). Mxr1p also regulates the expression of *GLN1* in cells cultured in YNB + Glu as well as YP media (Fig. 6*D*). *GLN1* was expressed from its own promoter as a Myc-tagged protein, and its localization in the cytoplasm was confirmed by immunofluorescence using anti-Myc tag antibodies (Fig. 6*E*). The function of *GLN1* could not be examined further as a Δ*gln1* strain could not be generated, despite several attempts. Thus, overexpression of Mxr1p in Δ*mxr1* strain results in the restoration of synthesis of several key enzymes involved in amino acid metabolism in cells cultured in YP as well as YNB + Glu media. In contrast, Mxr1p^{N400} restores the expression of only *AAT1* and *AAT2* in cells cultured in YP medium. It does not activate the transcription of any other target gene of Mxr1p in cells cultured

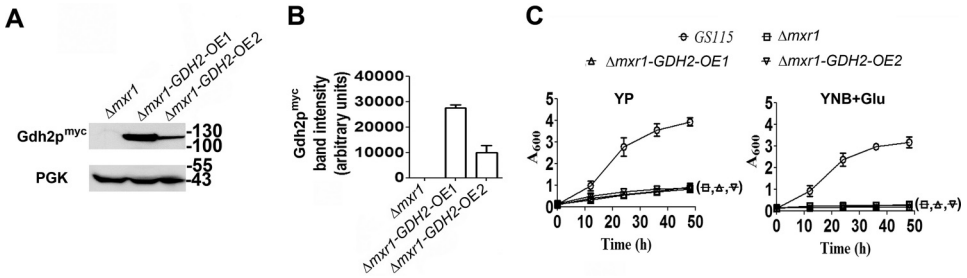


FIGURE 4. **Analysis of the ability of Gdh2p to restore the growth of Δ*mxr1*.** *A*, Western blotting analysis of lysates of Δ*mxr1*, Δ*mxr1* overexpressing *GDH2* from *GAPDH* promoter, or *CUP1* promoter. *B*, quantitation of data presented in *A*. *C*, analysis of ability of Gdh2p overexpressed from *GAPDH* or *CUP1* promoters to restore the growth of Δ*mxr1* cultured in YP and YNB + Glu media.

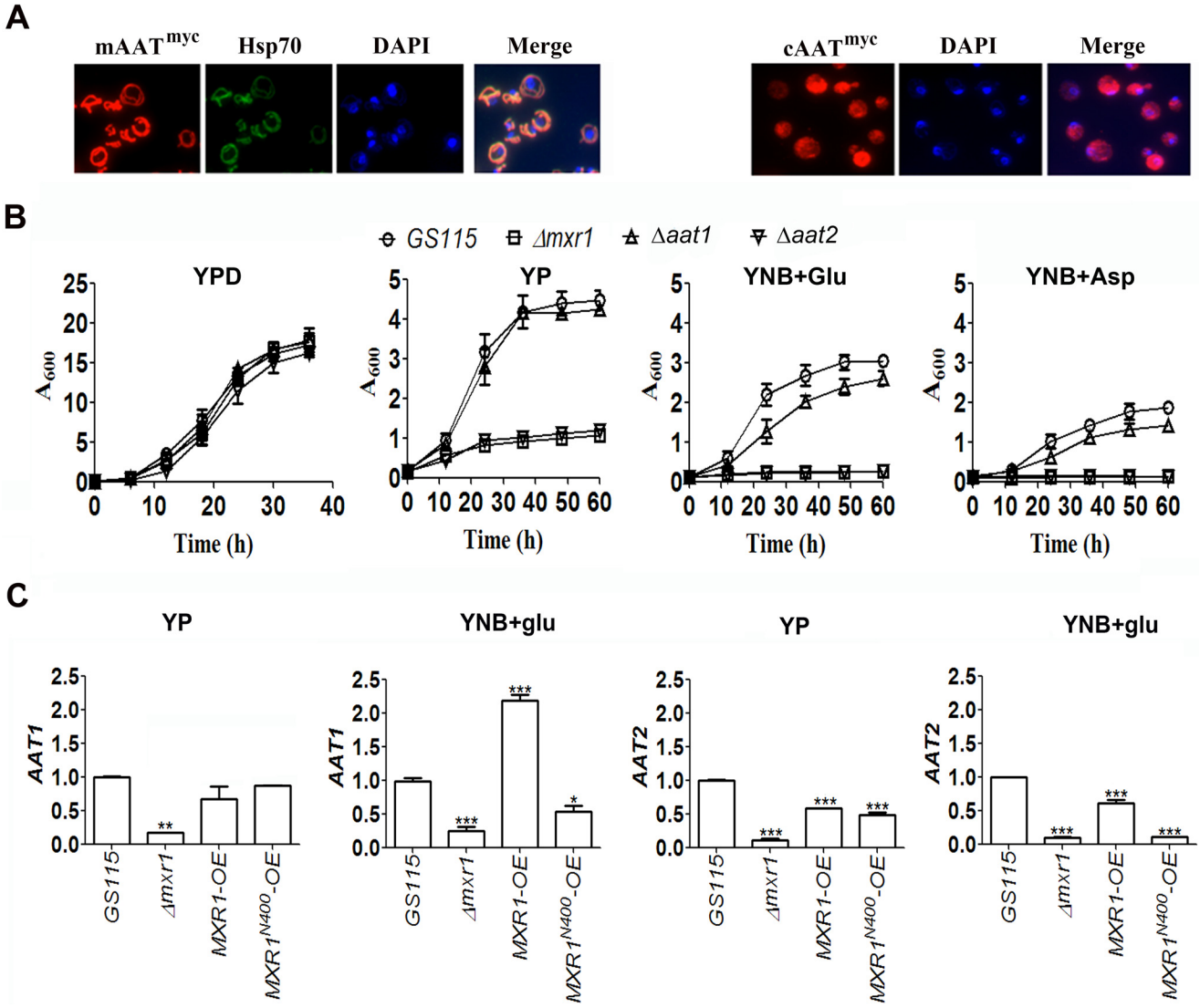
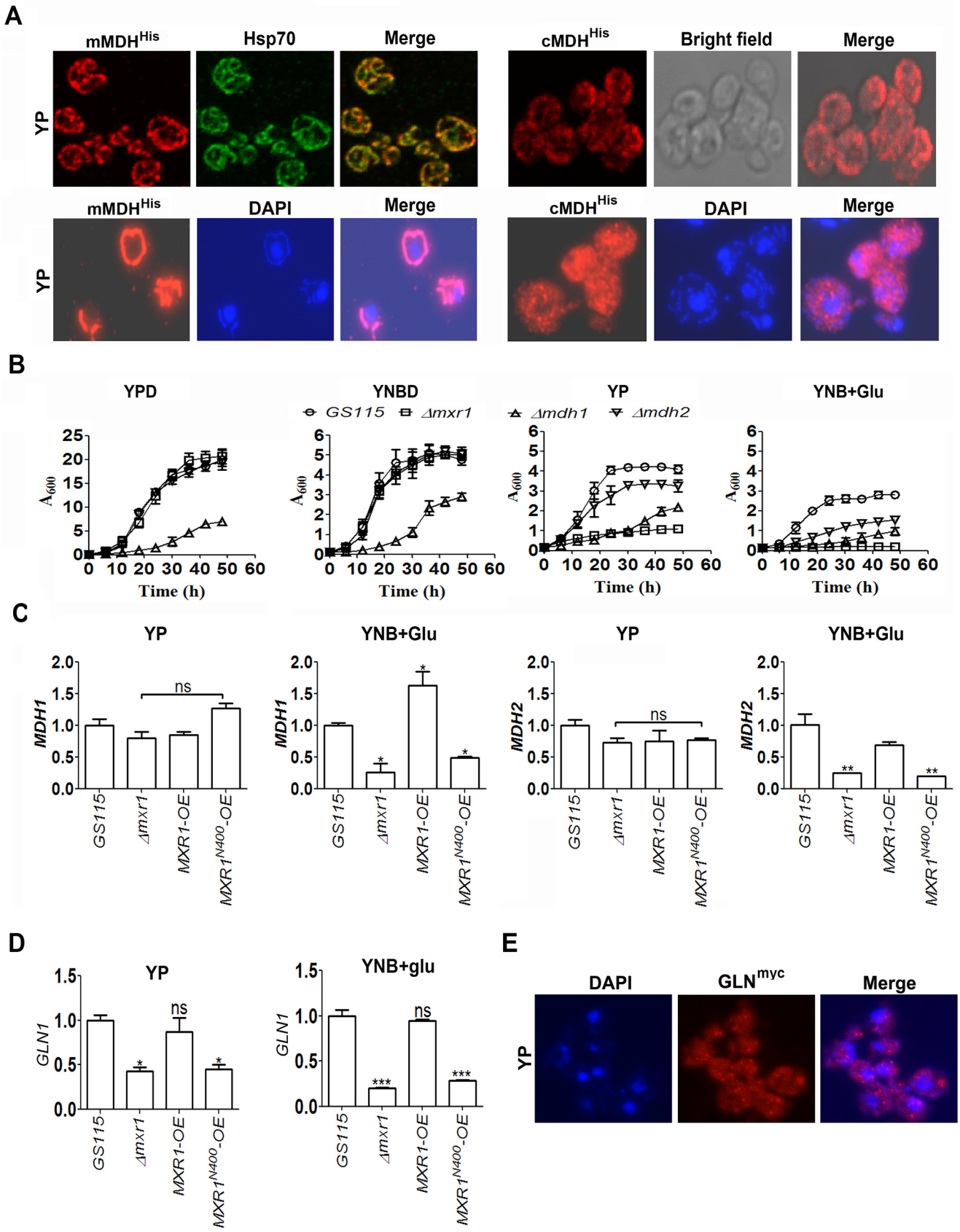


FIGURE 5. **Function and regulation of mAAT and cAAT encoded by *AAT1* and *AAT2*, respectively, in *P. pastoris* cells cultured in YP, YNB + Glu, and YP + Asp media.** *A*, localization of Myc-tagged mAAT and cAAT in the mitochondria and cytoplasm, respectively, by immunofluorescence using anti-Myc tag antibodies. Hsp70 and DAPI were used as mitochondrial and nuclear markers, respectively. *B*, effect of deletion of genes encoding cAAT and mAAT on the growth of *P. pastoris* in YP, YNB + Glu, and YP + Asp media. *C*, quantification of *AAT1* and *AAT2* mRNAs by qPCR in various *P. pastoris* strains as indicated. *Error bars* indicate mean ± S.D. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$. One-way analysis of variance followed by Tukey's multiple comparison test was carried out ($n = 3$).

in YNB + Glu medium. These results are summarized in Table 3.

Identification of Mxr1p Response Elements (MXREs) in the Promoters of AAT2, MDH2, and GLN1—Mxr1p regulates transcription of target genes by binding to *MXRE*s present in their promoters. The *MXRE*s identified so far contain a core motif whose consensus sequence is 5′-CYCCNY-3′ (Fig. 7*A*) (6, 7, 9, 13). A sequence similar to this core *MXRE* is present between −151 and −146 bp (site A) of the promoter of *AAT2*, −213 and −208 bp of the promoter of *MDH2*, and −528 and −523 bp of


A
mMDH^His
Hsp70
Merge
cMDH^His
Bright field
Merge
YP
mMDH^His
DAPI
Merge
cMDH^His
DAPI
Merge
YP
B
YPD
YNBD
GS115
Δmxr1
YP
Δmdh1
Δmdh2
YNB+Glu
A600
Time (h)
C
YP
YNB+Glu
YP
YNB+Glu
MDH1
MDH2
ns
GS115
Δmxr1
MXR1-OE
MXR1^N400-OE
D
YP
YNB+glu
GLN1
ns
GS115
Δmxr1
MXR1-OE
MXR1^N400-OE
E
DAPI
GLN^myc
Merge
YP

the promoter encoding *GLN1* (Fig. 7, *B*, *D*, and *E*). In the region between −118 and −113 bp (site B) of *AAT2* promoter, the sequence 5′-CCCCGA-3′ is present (Fig. 7*B*), which is similar to the consensus sequence 5′-CYCCNR-3′. Studies with *AOX1* promoter have shown that Mxr1p binds only to 5′-CYCCNY-3′ but not 5′-CYCCNR-3′ (6). To examine the ability of Mxr1p to bind to site A or site B of the *AAT2* promoter, oligonucleotides carrying mutations in either site B (*pAAT2-MXRE-M1*), site A (*pAAT2-MXRE-M2*), or both (*pAAT2-MXRE-M3*) were synthesized (Fig. 7*C*), and their ability to bind to recombinant His-tagged $Mxr1p^{N150}$ (6, 9) was examined by EMSA. Similarly, oligonucleotides carrying point mutations within the putative core *MXRE*s of promoters encoding *MDH2* and *GLN1* were synthesized (Fig. 7, *D* and *E*), and their ability to bind to recombinant His-tagged $Mxr1p^{N150}$ was examined by EMSA. The results indicate that $Mxr1p^{150}$ binds specifically to *pAAT2-MXRE* as well as *pAAT2-MXRE*-M1 but not *pAAT2-MXRE-M2* and *pAAT2-MXRE-M3* (Fig. 7*F*). Thus, Mxr1p binds specifically to site A but not site B of the *AAT2* promoter. Addition of anti-His tag antibodies resulted in the supershift of the DNA-protein complexes, confirming the presence of recombinant $Mxr1p^{N150}$ in these complexes (Fig. 7*F*). $Mxr1p^{N150}$ also binds specifically to *pMDH2-MXRE* and *pGLN1-MXRE* but not to *pMDH2-MXRE-M1* and *pGLN1-MXRE-M1*carrying point mutations within the core *MXRE*s (Fig. 7*F*).

Chromatin immunoprecipitation (ChIP) studies were carried out to study the ability of $Mxr1p^{N400}$ to bind to *MXRE*s present in the promoters of *AAT2* and *GLN1* in cells cultured in YP medium because $Mxr1p^{N400}$ activates the transcription of *AAT2* but not *GLN1* under these culture conditions (Table 3). Extracts were prepared from $MXR1^{N400}$*-OE* strain cultured in YP, and the presence of Myc-tagged $Mxr1p^{N400}$ was confirmed by Western blotting (Fig. 7*G*). $Mxr1p^{N400}$ was cross-linked to chromatin and immunoprecipitated with anti-Myc antibodies. DNA was extracted, and PCR was carried out with primers that amplify *AAT2* and *GLN1* promoter regions containing *MXRE*s (Fig. 7*H*). Only *AAT2* but not *GLN1* promoter was amplified by PCR (Fig. 7*I*) indicating that $Mxr1p^{N400}$ binds to the promoter of *AAT2* but not *GLN1* in cells cultured in YP medium. Thus, there is good correlation between promoter occupation *in vivo* and transcriptional activation by $Mxr1p^{N400}$ (Fig. 7*J*).

Discussion

The integration of carbon and nitrogen metabolism with energy production is crucial for the survival of living organisms. Although yeasts such as *S. cerevisiae* utilize amino acids primarily for nitrogen metabolism, respiratory yeasts such as *P. pastoris* and *P. stipitis* have evolved pathways for efficient utilization of amino acids both as a source of carbon and nitrogen. The generation of TCA cycle intermediates such as α-ketoglutarate and oxaloacetate from glutamate and aspartate is the first step in the utilization of amino acids as the sole source of carbon. While examining the role of Mxr1p in the regulation of various metabolic pathways, it was observed that *P. pastoris GS115* but not Δ*mxr1* can grow in media containing amino acids as the sole source of carbon. This led us to study the enzymes involved in the utilization of amino acids as well as to examine the role of Mxr1p in the regulation of their biosynthesis. We demonstrate that enzymes such as GDH2, cAAT, mAAT, cMDH, and mMDH, which play a crucial role in the inter-conversion of amino acids and keto acids in the cytosolic and mitochondrial compartments as well as glutamine synthetase involved in glutamine synthesis, are important for the utilization of amino acids as the sole source of carbon and nitrogen in *P. pastoris*. Disruption of genes encoding cAAT and cMDH impairs the ability of *P. pastoris* to grow in media containing amino acids as the sole source of carbon. Although mAAT is not required for growth in YP, its disruption results in growth retardation in YNB + Glu medium. Disruption of *MDH1* severely impairs growth in media containing not only amino acids but also glucose as the sole source of carbon.

An important aspect of this study is the identification of Mxr1p as a regulator of synthesis of key enzymes involved in the utilization of amino acids. Biosynthesis of AAT1, AAT2, MDH1, MDH2, and GLN1 is regulated by Mxr1p at the level of transcription as evident from the significant reduction in mRNA levels of genes encoding these enzymes in Δ*mxr1* strain. Promoters of *AAT2*, *MDH2*, and *GLN1* contain *MXRE*s to which recombinant Mxr1p binds specifically *in vitro*. Using *AAT2* and *GLN1* as examples, we have demonstrated the importance of promoter occupancy by Mxr1p *in vivo* for transcriptional activation of these genes. Key enzymes identified in this study, whose synthesis is regulated by Mxr1p, are depicted schematically in Fig. 8*A*. We conclude that Mxr1p functions as a global regulator of multiple metabolic pathways in *P. pastoris* (Fig. 8*B*).

Amino acids enhance growth rate as well as recombinant protein production in several yeast species when added to media containing conventional carbon sources, primarily by serving as precursors for the synthesis of proteins as well as participating in anaplerotic reactions (16–19). In the case of *P. pastoris*, mixed feeds of methanol and a multicarbon source instead of methanol as the sole carbon source have been shown to improve recombinant protein production (20). However, metabolic flux analysis during methanol metabolism in the presence of amino acids as a second source of carbon has not been examined thus far. The various *P. pastoris* strains described in this study can be exploited for these studies. Such

FIGURE 6. **Function and regulation of mMDH and cMDH in *P. pastoris* cells cultured in YP and YNB + Glu media.** *A*, localization of his-tagged mMDH and cMDH in the mitochondria and cytoplasm, respectively, by immunofluorescence using anti-His tag antibodies. The genes were expressed from their own promoters. Hsp70 was used as mitochondrial marker. DAPI was used as nuclear as well as mitochondrial marker. *B*, effect of deletion of genes encoding mMDH and cMDH on the growth of *P. pastoris* in YPD, YNB + 2% glucose (YNBD), YP, and YNB + Glu media. *C* and *D*, quantification of *MDH1*, *MDH2*, and *GLN1* mRNAs by qPCR in various *P. pastoris* strains as indicated. *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 carried out ($n = 3$). *E*, localization of Myc-tagged glutamine synthetase (*GLN*) in the cytoplasm by immunofluorescence using anti-Myc tag antibodies. *GLN1* was expressed from its own promoter. DAPI was used to stain nuclei.

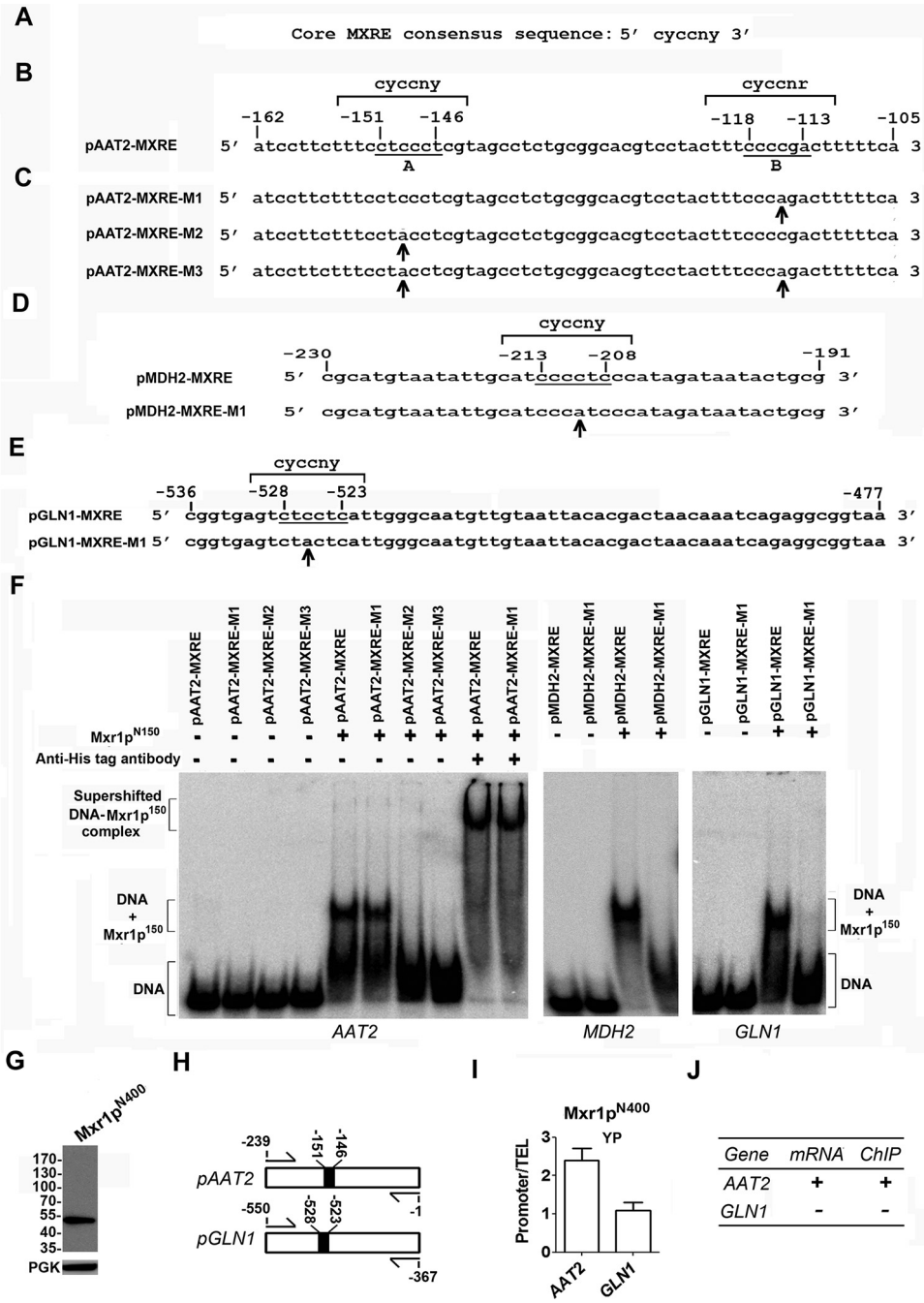


FIGURE 7. **Analysis of Mxr1p binding to *MXREs* present in the promoters of target genes by EMSA and ChIP.** *A,* consensus sequence of core *MXRE* (6, 7, 13). *B,* nucleotide sequence of *AAT2* promoter region between −105 and −162 bp. The nucleotide sequence of site A exactly matches with the *pAOXI-MXRE* consensus sequence (6) and that of site B differs by one nucleotide. *C,* nucleotide sequence of *pAAT2-MXRE* oligonucleotides used in EMSA. Mutated bases are indicated by *arrows*. *D,* oligonucleotides encompassing the *MDH2* promoter region between −191 and −230 bp. The nucleotide sequence between −208 and −213 bp exactly matches with the *MXRE* consensus sequence. Mutated base is indicated by an *arrow*. *E,* oligonucleotides encompassing the *GLN1* promoter region between −477 and −536 bp. The nucleotide sequence between −528 and −523 bp exactly matches with the *MXRE* consensus sequence. Mutated base is indicated by an *arrow*. *F,* EMSA with radiolabeled oligonucleotides and histidine-tagged recombinant Mxr1p containing 150 N-terminal amino acids encompassing the DNA binding domain ($Mxr1p^{150}$) as indicated. $Mxr1p^{150}$ has been described (6, 7). Supershift of DNA-$Mxr1p^{150}$ complex by the addition of anti-His antibodies is shown. *G,* Western blotting analysis of lysate of *$MXR1^{N400}$-OE* strain cultured in YP medium using anti-Myc antibodies. *H,* schematic representation of location of primers used in the PCRs for the amplification of promoter regions of *AAT2* and *GLN1* following ChIP. ChIP was carried out using anti-Myc antibody. *Black boxes* indicate core *MXRE*s (5′-CYCCNY-3′). *I,* analysis of enrichment of ChIP-ed DNA over the reference sample by qPCR (21, 22). The data are expressed as $Mxr1p^{N400}$ binding (ChIP/input) to *AAT2* and *GLN1* relative to binding (ChIP/input) at the *TEL* (telomere) region. *TEL* was used as a reference (14). Input refers to the amount of chromatin used in ChIP. The data represent the average of two independent experiments. *J, table* depicting correlation between promoter occupation by $Mxr1p^{N400}$ *in vivo* and transcript levels of target genes (Table 3) in *$MXR1^{N400}$-OE* strain cultured in YP medium.

studies may provide new insights into the potential interactions between methanol metabolism and amino acid metabolism on heterologous protein production, leading to novel biotechnological applications.

A surprising result of this study is the regulation of *GDH2* expression by Mxr1p at the translational level rather than the transcriptional level as evident from the reduction in GDH2 protein but not *mRNA* levels in the Δ*mxr1* strain. Furthermore,

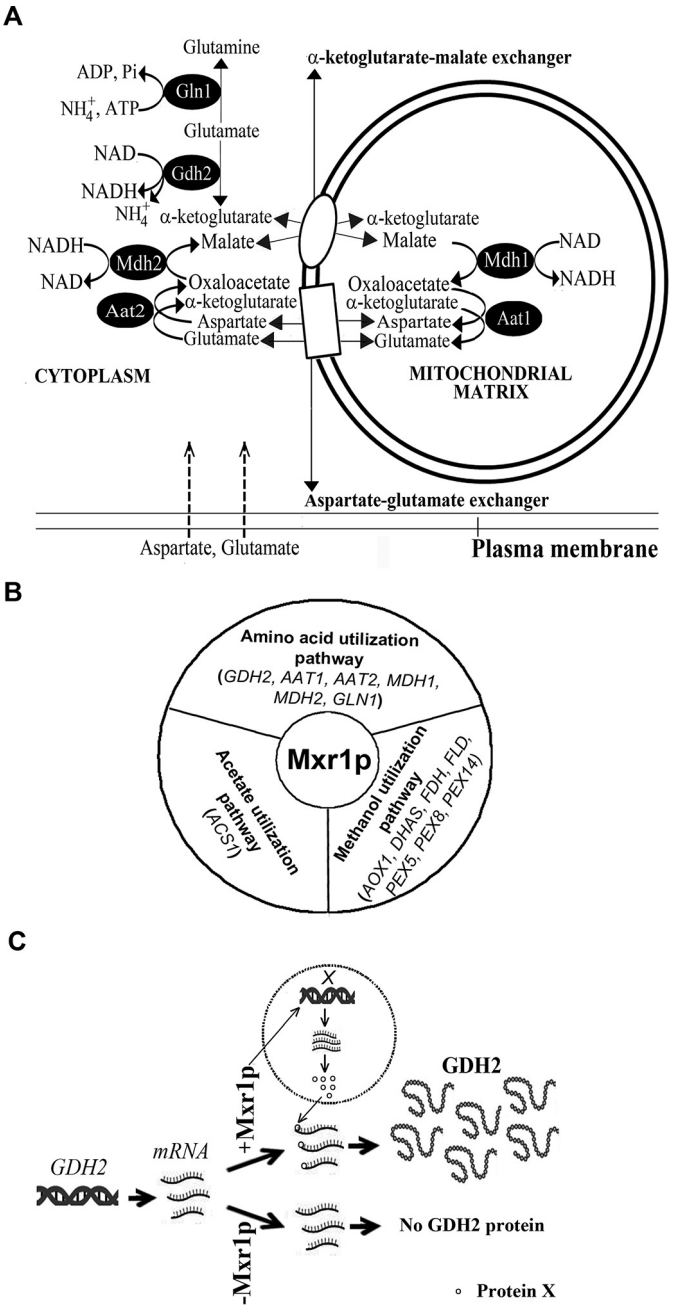


FIGURE 8. *A,* schematic representation of key enzymes involved in the utilization of amino acids as the sole source of carbon in *P. pastoris* whose expression is regulated by Mxr1p. *B,* schematic diagram depicting Mxr1p as a global regulator of multiple metabolic pathways. Genes regulated by Mxr1p in various metabolic pathways are indicated. Mxr1p target genes in methanol and acetate utilization pathways have been described (5, 13). *C,* model for the translational regulation of *GDH2* by Mxr1p in cells cultured in YP or YNB + Glu media. We hypothesize that Mxr1p regulates the transcription of a gene encoding protein X, which is required for the translation of *GDH2* mRNA. See under "Discussion" for details.

expression of Mxr1p but not Mxr1p^{N400} in Δ*mxr1* restores GDH2 protein levels indicating that the N-terminal region of Mxr1p alone is not sufficient for the post-transcriptional regulation of *GDH2*. *GDH2* is the first example of a gene whose expression is regulated by Mxr1p at the post-transcriptional level (Fig. 8*C*). Mxr1p may not be directly involved in the regulation of translation of *GDH2* mRNA because it localizes to the nucleus of cells cultured in YP medium. We hypothesize that Mxr1p activates the transcription of a gene whose product (protein X) is required for the efficient translation of *GDH2* mRNA (Fig. 8*C*).

Experimental Procedures

Media and Culture Conditions—P. pastoris (*GS115, his*$^-$) was cultured in either minimal medium containing 0.17% yeast nitrogen base (YNB) without amino acids and 0.5% ammonium sulfate supplemented with 2.0% glucose (YNBD), 1.0% glutamate (YNB + Glu), or 1% aspartate (YNB + Asp) or nutrient-rich YP medium (1.0% yeast extract and 2.0% peptone) alone or YP medium containing 2.0% glucose (YPD) or 2% methanol (YPM). *S. cerevisiae BY4741* strain (Euroscarf) was cultured in YPD/YP medium. Yeast strains were grown at 30 °C in an orbital shaker at 180 rpm. For growth assays, colonies were first cultured overnight in YPD medium, then washed with sterile water, and shifted to different media with initial A_{600} of ~0.1. To assay growth in liquid medium, *P. pastoris* cells grown overnight in YPD were diluted in duplicate with fresh YPD to A_{600} of 0.1, and aliquots of cells were removed at regular intervals, and A_{600} was measured. *Escherichia coli DH5α* and *BL21* (DE3) strains were used for the isolation of recombinant plasmids and expression of recombinant proteins, respectively. Bacterial and yeast transformations were done by $CaCl_2$ and electroporation method (Gene Pulser, Bio-Rad) respectively.

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

Generation of P. pastoris Strain Expressing GDH2^{His} from Its Own Promoter—The gene encoding GDH2 along with 545 bp of its promoter was cloned into *pIB3* vector (Addgene) as a histidine-tagged protein (GDH2^{His}). *GDH2* along with −545 bp of promoter was amplified from genomic DNA of *P. pastoris* by the primer pair 5′-CCGGAATTCCTCTCATGTTCGATATTC-CAGCGGCTTTC-3′ and 5′-CCGCTCGAGCTA*ATGATGAT-GATGATGATG*CAATCCCCGAGACTTGTAC-3′. EcoRI and XhoI sites are underlined. The histidine tag-encoding region is shown in italics. The PCR product was digested with EcoRI and XhoI and cloned into the *pIB3* vector at the EcoRI and XhoI sites to obtain *pIB3-GDH2^{His}*. After linearization with BspHI, the plasmid was transformed to *P. pastoris GS115* and Δ*mxr1* strains by electroporation to obtain *GS115-GDH2^{His}* and Δ*mxr1-GDH2^{His}* strains, respectively.

Generation of P. pastoris Δmxr1 Strain Overexpressing Mxr1p, Mxr1p^{N400}, or GDH2^{myc}—P. pastoris Δ*mxr1* strain in which *MXR1* coding region was replaced by a Zeocin expression cassette has been described (13). *P. pastoris* Δ*mxr1* strain overexpressing full-length Mxr1p (*MXR1-OE*) or a truncated Mxr1p containing 400 N-terminal amino acids (*MXR1^{N400}-OE*) has been described (13). To generate Δ*mxr1* strain expressing Myc-tagged GDH2 (GDH2^{Myc}) from the *GAPDH* promoter, the gene encoding GDH2 was amplified by PCR from *P. pastoris* genomic DNA using the primer pair 5′-CCGGAA-TTCATTATGGTCGACAGATCCAAG-3′ and 5′-CGGGGT-ACCCAATCCCCGAGACTTGTAC-3′. EcoRI and KpnI sites

in the primers are underlined. The gene was cloned at the EcoRI and KpnI sites of the *pGAPZA* vector, and the recombinant plasmid thus obtained (*pGAPDH-GDH2^{myc}*) was transformed into Δ*mxr1* strain to obtain Δ*mxr1-GDH2-OE1*. The *pCUP1-GDH2* expression cassette containing *S. cerevisiae* copper-inducible promoter (p*CUP1*) (15) was generated by a series of overlapping PCRs. The *S. cerevisiae CUP1* promoter region between −460 and −1 bp was amplified by PCR from *S. cerevisiae* genomic DNA using the primer pair 5′-CCGGAATTCGCCGATCCCATTACCGAC-3′ (−460 to −443 bp of *CUP* promoter, EcoRI site is underlined) and 5′-GACACTTGGAGTCTGTCGACCAT*TTTATGTGATGATTGATTGATTGATTG*-3′ (region from +1 to +23 bp of *GDH2* and −32 to −1 bp of *CUP1* promoter (italics)). In another PCR, *GDH2* was amplified from *P. pastoris* genomic DNA using the primer pair 5′-*CAATCAATCAATCAATCATCACATAA*ATGGTCGACAGACTCCAAGTGTC-3′ (−32 to −1 bp of *CUP* promoter, +1 to +23 bp of *GDH2* (italics)) and 5′-CGGGGTACCCAATCCCCGAGACTTGTAC-3′ (BamHI site is underlined). The products of the two PCRs were used as templates in the final PCR and amplified using the primer pair 5′-CCGGAATTCGCCGATCCCATTACCAC-3′ and 5′-CGGGGTACCCAATCCCCGAGACTTGTAC-3′ (EcoRI and BamHI sites are underlined). The *pCUP1-GDH2* expression cassette thus obtained was digested with EcoRI and KpnI and cloned into *pGAPZA* vector to obtain *pCUP-GDH2^{myc}* plasmid. The recombinant plasmid was linearized with AvrII and transformed to *P. pastoris* Δ*mxr1* strain by electroporation to obtain Δ*mxr1-GDH2-OE2*.

Construction of P. pastoris Δ*gdh2,* Δ*aat1,* Δ*aat2,* Δ*mdh1, and* Δ*mdh2 Strains*—The coding regions of genes encoding GDH2, AAT1, AAT2, MDH1, and MDH2 were deleted by homologous recombination method, using resistance to Zeocin (Zeocin^{R}) as selection marker. The gene deletion constructs for each of these genes consisted of ~1 kb of promoter, ~1.2 kb of Zeocin expression cassette, and ~1 kb of 3′-flanking sequences. The promoters and 3′-flanking regions of respective genes were amplified by PCR from *P. pastoris GS115* genomic DNA, and the Zeocin expression cassette was amplified from *pGAPZA* (Invitrogen).

For generating *P. pastoris* Δ*gdh2* strain, −930 bp of *GDH2* promoter, 1.2 kb of coding region, and 1 kb of 3′-flanking region were amplified by PCR using the primer pair 5′-GCTGACCTATAGTTTGCTAGAACCGTTTTCTC-3′ (−930 to −898 bp of *GDH2* promoter) and GCTATGGTGTGTGGGGGATCCGCA*GGTAGTTTTAGTTTCTTTCTACTAATTG* 3′ (+962 to +985 bp of *pGAPZA* and −52 to −23 bp of *GDH2* promoter (italics)). In another PCR, the primer pair 5′-*CAATTAGTAGAAAGAAACTAAAACTACC*TGCGGATCCCCCACACACCATAGC-3′ (−52 to −24 bp of *GDH2* promoter (italics), +962 to +985 bp of *pGAPZA*) and 5′-*GATATCCCCATACTTTTCTTCTCTTT*CCTTGCTCACATGTTGGTCTCCAGCTTGC-3′ (+3124 to +3152 bp of 3′-flanking region of *GDH2* in reverse complement (italics) +2136 to +2160 bp in reverse complement of *pGAPZA*) was used. In the third PCR, the primer pair 5′-*GCAAGCTGGAGACCAACATGTGAGCA*AGGAAAGAGAAGAAAAGTATGGGGATATC-3′ (+2136 to +2160 bp of *pGAPZA* (italics) and 30 nucleotides of 3′ UTR from +3124 to +3152 bp of 3′-flanking region of *GDH2*) and 5′-CTTACTTCATTAAAAGAGGTCATTCTTCTGCTACTGC-3′ (+4071 to +4100 bp of 3′-flanking region of *GDH2*) were used, respectively. The three PCR products were purified and used as templates in the final PCR along with the primers 5′-GCTGACCTATAGTTTGCTAGAACCGTTTTCTC-3′ and 5′-CTTACTTCATTAAAAGAGGTCATTCTTCTGCTACTGC-3′ to generate the 3.2-kb *GDH2* deletion construct that was transformed into *P. pastoris GS115* by electroporation. Zeocin-resistant colonies were selected, and *GDH2* deletion was confirmed by PCR and Southern blotting.

For generating *P. pastoris* Δ*aat1* strain, 1.025 kb of *AAT1* promoter region was amplified by PCR using the primer pair 5′-CAATTGCAATGTGATACGGTGGTAC-3′ (−1025 to −1000 bp of *AAT1*) and 5′-*GCTATGGTGTGTGGGGGATCCGCA*GATGACTTGATATGGTCTGATTTGG-3′ (+962 to +985 bp of pGAPZA in reverse complement (italics), and 25 nucleotides of 5′ UTR from −25 to −1 bp of *AAT1* in reverse complement). The 1.2-kb Zeocin expression cassette was amplified by PCR using the primer pair 5′-*CCAAATCAGACCATATCAAGTCATC*TGCGGATCCCCCACACACCATAGC-3′ (−25 to −1 bp of *AAT1*promoter (italics), +962 to +985 bp of *pGAPZA*) and 5′-*CATTGGCAAGGTAGTCAACGTTTTGG*CTCACATGTTGGTCTCCAGCTTG-3′ (+1230 to +1255 bp of 3′-flanking region of *AAT1* in reverse complement (italics), +3124 to +3152 bp in reverse complement of *pGAPZA*). The 1.011 kb of 3′-flanking region of *AAT1* was amplified by PCR using the primer pair 5′-*CAAGCTGGAGACCAACATGTGAGC*CAAAACGTTGACTACCTTGCCAATG-3′ (3124 to 3152 bp of *pGAPZA* (italics), +1230 to +1255 bp of 3′-flanking region of *AAT1*) and 5′-AGATCTCTCAAATACGATGGGGTC-3′ (+2252 to +2275 bp of 3′-flanking region of *AAT1*). All three PCR products thus obtained were purified and used as templates in the final PCR along with the primers 5′-CAATTGCAATGTGATACGGTGGTAC-3′ and 5′-AGATCTCTCAAATACGATGGGGTC-3′ to generate the *AAT1* deletion construct that was transformed to *P. pastoris GS115*. Zeocin-resistant colonies were selected, and deletion of *AAT1* was confirmed by PCR using gene-specific primers.

For generating *P. pastoris* Δ*aat2* strain, 1.009 kb of *AAT2* promoter region was amplified by PCR using the primer pair 5′-TATCACCATCAACTCTCGTTGATCTTTG-3′ (−1009 to −982 bp of *AAT2*) and 5′-*GCTATGGTGTGTGGGGGATCCGCA*GCTTAACGGTATGTTGACGAGTTC-3′ (+962 to +985 bp of *pGAPZA* in reverse complement (italics), −24 to −1 bp in reverse complement of *AAT2*). In another PCR, the 1.2-kb Zeocin expression cassette was amplified by PCR using the primer pair 5′-*GAACTCGTCAACATACCGTTAAGC*TGCGGATCCCCCACACACCATAGC-3′ (−24 to −1 bp of *AAT2* (italics), +962 to +985 bp of *pGAPZA*) and 5′-*CTTATCGTTTATAAATCACTCGTT*GCTCACATGTTGGTCTCCAGCTTG-3′ (+1247 to +1270 bp in reverse complement of 3′-flanking region of *AAT2* (italics), +3124 to +3152 bp in reverse complement of *pGAPZA*). In the third PCR, 1.045 kb of 3′-flanking region of *AAT2* was amplified using the primer pair 5′-*CAAGCTGGAGACCAACATGTGAGC*AACGAGTGATTTATAAACGATAAG-3′ (+3124 to +3152 bp of *pGAPZA* (italics), +1247 to +1270 bp of 3′-flanking region of *AAT2*) and 5′-CTTCGTTGGTGAAAACCAAGATGCTTG-3′ (3′ UTR from +2257 to

+2231 bp of 3′-flanking region of *AAT2*). All three PCR products were purified and used as templates in a final PCR along with the primers 5′-TATCACCATCAACTCTCGTTGATCTTTG-3′ and 5′-CTTCGTTGGTGAAAACCAAGATGCTTG-3′. The 3.2-kb PCR product thus obtained consisting of a Zeocin expression cassette flanked by ~1 kb of *AAT2* promoter and 1 kb of 3′-flanking region of *AAT2* was transformed into *P. pastoris GS115*. Zeocin-resistant colonies were selected, and deletion of *AAT2* was confirmed by PCR using gene-specific primers.

For generating *P. pastoris* Δ*mdh1* strain, ~1 kb of *MDH1* promoter was amplified by PCR using the primer pair 5′-TGTGTAGCTCTGAACTCGTTG-3′ (−921 to −900 bp of *MDH1*) and 5′-*CTATGGTGTGTGGGGGATCCGCA*TGAAGGGATTATAATGGTTG-3′ (962 to 984 bp of *pGAPZA* in reverse complement (italics), −20 to −1 bp of *MDH1* in reverse complement). In another PCR, the Zeocin expression cassette was amplified by PCR using the primer pair 5′-*CAACCATTATAATCCCTTCA*TGCGGATCCCCCACACACCATAG-3′ (−20 to −1 bp of *MDH1* (italics), +962 to +984 bp of *pGAPZA*) and 5′-*CGTTCAATATCGTCACCCAGGA*GCTCACATGTTGGTCTCCAGC-3′ (+1060 to +1081 bp in reverse complement of 3′-flanking region of *MDH1* (italics), +3124 to +3147 bp in reverse complement of *pGAPZA*). In the third PCR, the 3′-flanking region of *MDH1* was amplified using the primer pair 5′-*GCTGGAGACCAACATGTGAGC*TCCTGGGTGACGATATTGAACG-3′ (+3130 to +3151 bp of *pGAPZA*, +1060 to +1081 bp of 3′-flanking region of *MDH1*) and 5′-CGTGGGGATGGACATGATCGTGG-3′ (+1997 to +2019 bp in the reverse complement of 3′-flanking region of *MDH1*). All three PCR products were purified and used as templates in the final PCR along with the primer pair 5′-CGTGGGGATGGACATGATCGTGG-3′ and 5′-TGTGTAGCTCTGAACTCGTTG-3′ to obtain a 3.2-kb product consisting of Zeocin expression cassette flanked by ~1 kb of *MDH1* promoter and 1 kb of 3′-flanking region of *MDH1* that was transformed into *P. pastoris GS115*. Zeocin-resistant colonies were selected, and deletion of *MDH1* was confirmed by PCR using gene-specific primers.

For generating *P. pastoris* Δ*mdh2* strain, ~1-kb *MDH2* promoter was amplified by PCR using the primer pair 5′-GAGGACGTGTGGGATTAGAAAG-3′ (−1047 to −1026 bp of *MDH2*) and 5′-*CTATGGTGTGTGGGGGATCCGCA*TTTGTTATTGTTCGTTGGTTGTAAC-3′ (+962 to +985 bp of *pGAPZA* in reverse complement (italics), −24 to −1 bp in reverse complement of *MDH2*). The Zeocin expression cassette was amplified by PCR using the primer pair 5′-*GTTACAACCAACGAACAATAACAAA*TGCGGATCCCCCACACACCATAG-3′ (−24 to −1 bp of *MDH2* (italics), +962 to +984 bp of *pGAPZA*) and 5′-*GAGACCAGCCCAGAGTGCACAAT*GCTCACATGTTGGTCTCCAGC-3′ (+1232 to +1054 bp in reverse complement of 3′-flanking region of *MDH2* (italics), +3124 to +3152 bp in reverse complement of *pGAPZA*). In the third PCR, the 3′-flanking region of *MDH2* was amplified using the primer pair 5′-*GCTGGAGACCAACATGTGAGC*ATTGTGCACTCTGGGCTGGTCTC-3′ (+3130 to +3151 bp of *pGAPZA* (italics), +2017 to +2040 bp of 3′-flanking region of *MDH2*) and 5′-AATATGCTAGTCATGTGACCATAG-3′ (+2017 to +2040 bp in reverse complement of 3′-flanking region of *MDH2*). All three PCR products were purified and used as templates in a final PCR along with the primer pair 5′-GAGGACGTGTGGGATTAGAAAG-3′ and 5′-AATATGCTAGTCATGTGACCATAG-3′. The 3.2-kb PCR product thus obtained consisting of Zeocin expression cassette flanked by ~1 kb of *MDH2* promoter and 1 kb of 3′-flanking region of *MDH2* was transformed into *P. pastoris GS115*. Zeocin-resistant colonies were selected, and deletion of *MDH2* was confirmed by PCR using gene-specific primers.

Generation of P. pastoris Strains AAT1^{Myc}, AAT2^{Myc}, MDH1^{His}, MDH2^{His}, and GLN1^{Myc}—Expression cassettes consisting of genes encoding AAT1/AAT2 or MDH1/MDH2 along with ~1 kb of their own promoters were cloned into *pGAPZA* or *pIB3* vector (Addgene) and expressed in *P. pastoris GS115* as Myc- or His-tagged proteins. *AAT1* and *AAT2* encoding mitochondrial (mAAT) and cytosolic AAT (cAAT), respectively, were cloned into *pGAPZA* vector, whereas *MDH1* and *MDH2* encoding mitochondrial MDH (mMDH) and cytosolic MDH (cMDH), respectively, were cloned into *pIB3* vector. *AAT1* was amplified from *P. pastoris GS115* genomic DNA by the primer pair 5′-CGGGGTACCCAATTGCAATGTGATACGGTGGTAC-3′ and 5′-ATAAGAATGCGGCCGCTGTTGGTCGTAACCTCGTGG-3′. The KpnI and NotI sites in the primers are underlined. Because *AAT2* had an intron from +716 to +800 bp, *AAT2* cDNA was obtained by RT-PCR from RNA. The AAT2 expression cassette was generated as follows: *AAT2* promoter (−1000 bp) was amplified by PCR using the primer pair 5′-CGGGGTACCTATCACCATCAACTCTCGTTGATCTTTG-3′ (KpnI site is underlined) and 5′-GATATTCTGCGTTGAAAACGACAT*GCTTAACGGTATGTTGACGAGTTC*-3′ (+1 to +24 bp of *AAT2*, −24 to −1 bp of *AAT2* in italics) from genomic DNA. *AAT2* coding region was obtained by RT-PCR from *P. pastoris* RNA and the primer pair 5′-*GAACTCGTCAACATACCGTTAAGC*ATGTCGTTTTCAACGCAGAATATC-3′ (−24 to −1 bp of *AAT2* in italics, +1 to +24 bp of *AAT2*) and 5′-ATAAGAATGCGGCCGCTTACACGCACAACCTGATCAATG-3′ (NotI site is underlined). Products of the two PCRs were purified and used as templates in the final PCR along with the primer pair, 5′-CGGGGTACCTATCACCATCAACTCTCGTTGATCTTTG-3′ and ATAAGAATGCGGCCGCTTACACGCACAACCTGATCAATG-3′. *AAT1* and *AAT2* expression cassettes were cloned into the KpnI and NotI sites of *pGAPZA* vector to obtain *pGAPZA-AAT1^{Myc}* and *pGAPZA-AAT2^{Myc}* vectors, respectively. These plasmids were transformed into *P. pastoris GS115* to generate *AAT1^{Myc}* and *AAT2^{Myc}*, respectively.

MDH1 was amplified by PCR with the primer pair 5′-CGCGGATCCTGTGTAGCTCTGAACTCGTTG-3′ (EcoRI site is underlined) and 5′-CCGCTCGAGCTA*ATGATGATGATGATGATG*TGGGTTTTGCTTAACAAACTC-3′ (XhoI site underlined, DNA encoding His tag is shown in italics). *MDH2* was amplified by PCR using the primer pair 5′-CGCGGATCCGAGGACGTGTGGGATTAGAAAG-3′ (EcoRI site is underlined) and 5′-CCGCTCGAGCTA*ATGATGATGATGATGATG*GTTGCCAGCAATGAAGGCAGTTC-3′ (XhoI site is underlined; DNA encoding His tag is shown in italics). *P. pastoris* genomic DNA was used as the template. The *MDH1* and *MDH2* expression cassettes were digested with EcoRI and XhoI

TABLE 2
Plasmids used in this study

Plasmid	Description	Source/Ref.
pIB3	*P. pastoris* expression vector with *HIS4* selection marker	Addgene
pIB3-GDH2^{His}	*pIB3* expressing *GDH2*^{His} from − 545 bp of *P. pastoris GDH2* promoter	This study
pGAPZA	*P. pastoris* expression vector with *GAPDH* promoter and Zeocin^{R} selection marker	Invitrogen
pGAPBA	Modified *pGAPZA* vector with *GAPDH* promoter and Blasticidin^{R} selection marker	13
pGAPDH-GDH2^{Myc}	*pGAPZA* vector expressing Myc-tagged GDH2 from *GAPDH* promoter	This study
pCUP-GDH2^{Myc}	*pGAPZA* vector expressing Myc-tagged GDH2 from *CUP1* promoter	This study
pGAPBA-MXR1^{Myc}	*pGAPBA* vector expressing Mxr1p from *GAPDH* promoter	13
pGAPBA-MXR1^{N400}	*pGAPBA* vector expressing Mxr1p^{N400} from *GAPDH* promoter	13
pGAPZA-AAT1^{Myc}	*pGAPZA* vector expressing Myc-tagged AAT1 from − 1000 bp of *AAT1* promoter	This study
pGAPZA-AAT2^{Myc}	*pGAPZA* vector expressing Myc-tagged AAT2 from − 1000 bp of *AAT2* promoter	This study
pIB3-MDH1^{His}	*pIB3* vector expressing His-tagged MDH1 from − 1000 bp of *MDH1* promoter	This study
pIB3-MDH2^{His}	*pIB3* vector expressing His-tagged MDH2 from − 1000 bp of *MDH2* promoter	This study
pGAPZA-GLN1^{Myc}	*pGAPZA* vector expressing Myc-tagged GLN1 from − 1000 bp of *GLN1* promoter	This study

TABLE 3
Restoration of mRNA/protein levels of target genes in Δ*mxr1* strain by Mxr1p/Mxr1p^{N400} in cells cultured in YP or YNB + Glu media

Transcription factor	Target genes					
	GDH2[a]	*AAT1*	*AAT2*	*MDH1*	*MDH2*	*GLN1*
YP medium						
Mxr1p	+	+	+	−	−	+
Mxr1p^{N400}	−	+	+	−	−	−
YNB + Glu medium						
Mxr1p	+	+	+	+	+	+
Mxr1p^{N400}	−	−	−	−	−	−

[a] Mxr1p regulates the synthesis of GDH2 protein but not *GDH2* mRNA.

and cloned into *pIB3* vector to obtain *pIB3-MDH1*^{His} and *pIB3-MDH2*^{His}, respectively. These plasmids were transformed into *P. pastoris GS115* to obtain *MDH1*^{His} and *MDH2*^{His} strains, respectively.

GLN1 along with −1000 bp of its own promoter was amplified by PCR with primer pairs 5′-CCGCTCGAGTCAGTATTAATCTTGTCACATGACCTAC-3′ and 5′-TAAGAATGCGGCCGCTATCAGATTCTCTCTTGTACTCCTTG-3′ from *P. pastoris* genomic DNA. KpnI and NotI restriction sites are underlined. PCR product was digested with KpnI and NotI and cloned into *pGAPZA* vector. The recombinant plasmid (*pGAPZA-GLN1*^{Myc}) was linearized and transformed to *GS115* to obtain *GLN1*^{Myc} strain. *P. pastoris* strains and plasmids used in this study are listed in Tables 1 and 2, respectively.

Western Blotting—Total protein extracts were prepared from *P. pastoris* by vortexing with glass beads, and cell lysates containing 50 μg of proteins were resolved by PAGE in the presence of SDS. The resolved proteins were transferred to a PVDF membrane using an electroblotting apparatus in transfer buffer (39 mM glycine, 48 mM Tris-HCl (pH 8.0), 20% methanol). The membrane was blocked overnight in 5% nonfat milk (HiMedia) prepared in 1× TBS (25 mM Tris-HCl, 125 mM NaCl (pH 8.0)). The protein of interest was detected by using antibodies raised against the specific protein or anti-epitope tag antibodies. Primary antibodies were detected by peroxidase-conjugated anti-rabbit/anti-mouse IgG secondary antibody (1:10,000 dilutions; Bangalore Genei, India). The blots were developed with chemiluminescence plus reagents (PerkinElmer Life Sciences) according to the manufacturer's instructions.

Subcellular Localization—Subcellular localization of epitope-tagged proteins was studied by immunofluorescence using a fluorescent microscope (Leica) and Zeiss confocal microscope (Zeiss 510 Meta). Immunofluorescence was carried out essentially as described (8).

Quantitative Real Time-PCR (qPCR)—RNA isolation from *P. pastoris* cells was carried out as described previously (8). qPCR was performed using iQ SYBR Green Super Mix and a iQ5 multi-Color real time PCR thermal cycler (iCycler, Bio-Rad). Levels of mRNA in mutant *P. pastoris* strains relative to *GS115* were normalized to 18S mRNA. Data were analyzed by the comparative *Ct* method for relative quantification (ΔΔ*Ct* method), which describes the change in expression of the target genes in a test sample relative to a calibrator sample. The following primer pairs were used in qPCRs: *MXR1,* 5′-TGCTGAAACTTGGATGAAC-3′ and 5′-TCGGATATAATATAGGCTCTGAAT-3′; *GDH2,* 5′-CCGCAGTTGGTATGTTC-3′ and 5′-CCTCTTGGTGTGATTGGA-3′; *AAT1,* 5′-CTCTACAACCAGGACACTAA-3′ and 5′-CATGGCTTACCATTCTCATC-3′; *AAT2,* 5′-TTGGCTGGATTCTTGGAT-3′ and 5′-ATAGAGGAAGATGCTGGTAC-3′; *MDH1,* 5′-TCTCAATCTAAGCACAAGGA-3′ and 5′-GGCTCAACGACATCATTC-3′; *MDH2,* 5′-AGGAGTCGGTAAGGATCTA and 5′-TTAGCCAAGTCTCTGATGAT-3′; *GLN1,* 5′-TTCCATCCTAAGCCACTG and 5′-TCGTTGTCAGAACCATATAGA-3′; *18S rRNA,* 5′-AATGAGGATTGACAGGATGA-3′ and 5′-AGGTCTCGTTATCG-3′.

Statistical Analysis—Statistical tests were carried out by one-way analysis of variance followed by Tukey's multiple comparison. GraphPad Prism 5 software was used. Data are presented as mean ± S.D. *p* value summary is mentioned on the bar of each figure as follows: *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$, *ns,* not significant.

DNA-Protein Interactions—Recombinant Mxr1p^{N150} consisting of 150 N-terminal amino acids of Mxr1p was expressed as His-tagged protein in *E. coli* and purified essentially as described (5). The ability of Mxr1p^{N150} to bind to ^{32}P-labeled oligonucleotides containing MXREs of promoters encoding AAT2, MDH2, and GLN1 was examined by electrophoretic

mobility shift assay (EMSA) essentially as described previously (5).

Chromatin Immunoprecipitation (ChIP)—ChIP was performed essentially as described (21). The following primer pairs were used for qPCR: *AAT2,* 5′-GTTTATCCGCTTGGACCGTG-3′ (−239 to −219 bp of *AAT2* promoter) and 5′-GCTTAACGGTATGTTGACGAGTTC-3′ (−24 to −1 bp of *AAT2* promoter); *GLN1,* 5′-GAACGACCATTTACTTTTTTAGATAGGC-3′ (−550 to −576 bp of *GLN1* promoter) and 5′-GAACTTTCTGGAGAAGAAACGCTG-3′ (−390 to −367 bp of *GLN1* promoter); *TEL*, 5′-CAGGGCCAGATGGAAAAATA-3′ and 5′-AACCGTTTACTACCGCATGG-3′ (14). ChIP qPCR results were analyzed based on the methodology described by Chakrabarti *et al.* (22). Binding of Mxr1p to the promoters of *AAT2* and *GLN1* was normalized with input. Telomeric (*TEL*) DNA was taken as control for the nonspecific DNA binding region (14).

Author Contributions—U. S. and P. N. R. designed the study, and U. S. performed all the experiments. P. N. R. wrote the paper. U. S. and P. N. R. analyzed the results and approved the final version of the manuscript.

References

1. Magasanik, B. (2003) Ammonia assimilation by *Saccharomyces cerevisiae. Eukaryot. Cell* **2,** 827–829
2. Miller, S. M., and Magasanik, B. (1990) Role of NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae. J. Bacteriol.* **172,** 4927–4935
3. Freese, S., Vogts, T., Speer, F., Schäfer, B., Passoth, V., and Klinner, U. (2011) C- and N-catabolic utilization of tricarboxylic acid cycle-related amino acids by *Scheffersomyces stipitis* and other yeasts. *Yeast* **28,** 375–390
4. Easlon, E., Tsang, F., Skinner, C., Wang, C., and Lin, S. (2008) The malate-aspartate NADH shuttle components are novel metabolic longevity regulators required for calorie restriction-mediated life span extension in yeast. *Genes Dev.* **22,** 931–944
5. Lin-Cereghino, G. P., Godfrey, L., de la Cruz, B. J., Johnson, S., Khuongsathiene, S., Tolstorukov, I., Yan, M., Lin-Cereghino, J., Veenhuis, M., Subramani, S., and Cregg, J. M. (2006) Mxr1p, a key regulator of the methanol utilization pathway and peroxisomal genes in *Pichia pastoris. Mol. Cell. Biol.* **26,** 883–897
6. Kranthi, B. V., Kumar, R., Kumar, N. V., Rao, D. N., and Rangarajan, P. N. (2009) Identification of key DNA elements involved in promoter recognition by Mxr1p, a master regulator of methanol utilization pathway in *Pichia pastoris. Biochim. Biophys. Acta* **1789,** 460–468
7. Kranthi, B. V., Kumar, H. R., and Rangarajan, P. N. (2010) Identification of Mxr1p-binding sites in the promoters of genes encoding dihydroxyacetone synthase and peroxin 8 of the methylotrophic yeast *Pichia pastoris. Yeast* **27,** 705–711
8. Kumar, N. V., and Rangarajan, P. N. (2011) Catabolite repression of phosphoenolpyruvate carboxykinase by a zinc finger protein under biotin and pyruvate carboxylase-deficient conditions in *Pichia pastoris. Microbiology* **157,** 3361–3369
9. Kumar, N. V., and Rangarajan, P. N. (2012) The zinc finger proteins Mxr1p and repressor of phosphoenolpyruvate carboxykinase (ROP) have the same DNA binding specificity but regulate methanol metabolism antagonistically in *Pichia pastoris. J. Biol. Chem.* **287,** 34465–34473
10. Sahu, U., Krishna Rao, K., and Rangarajan, P. N. (2014) Trm1p, a Zn(II)2Cys6-type transcription factor, is essential for the transcriptional activation of genes of methanol utilization pathway, in *Pichia pastoris. Biochem. Biophys. Res. Commun.* **451,** 156–164
11. Wang, X., Cai, M., Shi, L., Wang, Q., Zhu, J., Wang, J., Zhou, M., Zhou, X., and Zhang, Y. (2016) PpNrg1 is a transcriptional repressor for glucose and glycerol repression of AOX1 promoter in methylotrophic yeast *Pichia pastoris. Biotechnol. Lett.* **38,** 291–298
12. Wang, X., Wang, Q., Wang, J., Bai, P., Shi, L., Shen, W., Zhou, M., Zhou, X., Zhang, Y., and Cai, M. (2016) Mit1 transcription factor mediates methanol signalling and regulates alcohol oxidase 1 promoter in *Pichia pastoris. J. Biol. Chem.* **291,** 6245–6261
13. Sahu, U., and Rangarajan, P. N. (2016) Regulation of acetate metabolism and acetyl Co-A synthetase 1 (ACS1) expression by methanol expression regulator 1 (Mxr1p) in the methylotrophic yeast, *Pichia pastoris. J. Biol. Chem.* **291,** 3648–3657
14. Parua, P. K., Ryan, P. M., Trang, K., and Young, E. T. (2012) *Pichia pastoris* 14-3-3 regulates transcriptional activity of the methanol inducible transcription factor Mxr1 by direct interaction. *Mol. Microbiol.* **85,** 282–298
15. Mascorro-Gallardo, J. O., Covarrubias, A. A., and Gaxiola, R. (1996) Construction of a CUP1 promoter-based vector to modulate gene expression in *Saccharomyces cerevisiae. Gene* **172,** 169–170
16. Bakker, B. M., Overkamp, K. M., van Maris, A. J., Kötter, P., Luttik, M. A., van Dijken, J. P., and Pronk, J. T. (2001) Stoichiometry and compartmentation of NADH metabolism in *Saccharomyces cerevisiae. FEMS Microbiol. Rev.* **25,** 15–37
17. Görgens, J. F., van Zyl, W. H., Knoetze, J. H., and Hahn-Hägerdal, B. (2005) Amino acid supplementation improves heterologous protein production by *Saccharomyces cerevisiae* in defined medium. *Appl. Microbiol. Biotechnol.* **67,** 684–691
18. Görgens, J. F., Passoth, V., van Zyl, W. H., Knoetze, J. H., and Hahn-Hägerdal, B. (2005) Amino acid supplementation, controlled oxygen limitation and sequential double induction improves heterologous xylanase production by *Pichia stipitis. FEMS Yeast Res.* **5,** 677–683
19. Heyland, J., Fu, J., Blank, L. M., and Schmid, A. (2011) Carbon metabolism limits recombinant protein production in *Pichia pastoris. Biotechnol. Bioeng.* **108,** 1942–1953
20. Jordà, J., Jouhten, P., Cámara, E., Maaheimo, H., Albiol, J., and Ferrer, P. (2012) Metabolic flux profiling of recombinant protein secreting *Pichia pastoris* growing on glucose:methanol mixtures. *Microb. Cell Fact.* **11,** 57
21. Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D., and Broach, J. R. (1993) Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* **7,** 592–604
22. Chakrabarti, S. K., James, J. C., and Mirmira, R. G. (2002) Quantitative assessment of gene targeting *in vitro* and *in vivo* by the pancreatic transcription factor, Pdx1. Importance of chromatin structure in directing promoter binding. *J. Biol. Chem.* **277,** 13286–13293
23. Kranthi, B. V., Balasubramanian, N., and Rangarajan, P. N. (2006) Isolation of a single-stranded DNA-binding protein from the methylotrophic yeast, *Pichia pastoris* and its identification as ζ crystallin. *Nucleic Acids Res.* **34,** 4060–4068