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Trm1p, a Zn(II)$_2$Cys$_6$-type transcription factor, is essential for the transcriptional activation of genes of methanol utilization pathway, in *Pichia pastoris*

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ABSTRACT

The zinc finger transcription factors Mxr1p and Rop are key regulators of methanol metabolism in the methylotrophic yeast, *Pichia pastoris*, while Trm1p and Trm2p regulate methanol metabolism in *Candida boidinii*. Here, we demonstrate that Trm1p is essential for the expression of genes of methanol utilization (mut) pathway in *P. pastoris* as well. Expression of *AOXI* and other genes of mut pathway is severely compromised in *P. pastoris ΔTrm1* strain resulting in impaired growth on media containing methanol as the sole source of carbon. Trm1p localizes to the nucleus of cells cultured on glucose or methanol. The zinc finger domain of Mxr1p but not Trm1p binds to *AOXI* promoter sequences *in vitro*, indicating that these two positive regulators act by different mechanisms. We conclude that both Trm1p and Mxr1p are essential for the expression of genes of mut pathway in *P. pastoris* and the mechanism of transcriptional regulation of mut pathway may be similar in *P. pastoris* and *C. boidinii*.

1. Introduction

Methylotrophic yeasts such as *Pichia pastoris*, *Candida boidinii* and *Hansenula polymorpha* possess a well characterized methanol utilization (mut) pathway [1]. In these yeasts, expression of genes of mut pathway are repressed when cells are cultured in a medium containing glucose and induced when methanol is the sole source of carbon. The promoters of methanol-inducible genes such as alcohol oxidase I (AOXI) have been exploited for the expression of several heterologous proteins [2–5]. Several *cis*-acting elements required for methanol-inducible expression have been identified in the promoters of genes of mut pathway [6–14,1,15].

Transcription factors belonging to Zn(II)$_2$Cys$_6$- and C$_2$H$_2$-zinc finger family play a key role in the regulation of expression of genes of mut pathway of methylotrophic yeasts. A Zn(II)$_2$Cys$_6$-type transcription factor known as Mpp1p in *H. polymorpha* and Trm1p in *C. boidinii*, are essential for the expression of genes of mut pathway including those involved in peroxisome biogenesis [16,17]. In addition to Trm1p, Trm2p, a C$_2$H$_2$-type zinc finger protein, is also essential for the expression of genes of mut pathway in *C. boidinii* [18]. In *P. pastoris*, two zinc finger proteins, Mxr1p (Trmp2 homologue) and Rop (referred to as Rop1p in this study) function as positive and negative regulators of genes of mut pathway, respectively [9–12]. The function of Trm1p has not been investigated in detail in *P. pastoris*. Here, we demonstrate that Trm1p is essential for the expression of genes of mut pathway in *P. pastoris*. Our results indicate that both Mxr1p and Trm1p are required for the transcriptional activation of genes of mut pathway in *P. pastoris* and the mechanism of transcriptional regulation of methanol metabolism in *P. pastoris* is similar to that reported for *C. boidinii*.

2. Materials and methods

2.1. Yeast and bacterial strains & vectors

Escherichia coli strains DH5α and BL21(DE3)pLysS were purchased from Amersham Biosciences, Uppasala, Sweden. *E. coli* expression vector pRSETA were obtained from Invitrogen, USA. *P. pastoris* GS115 and *ΔMxr1* strains were obtained from James Cregg [9].

2.2. Media & culture conditions

P. pastoris was cultured at 30 °C in shake flasks containing either minimal (YNB) or nutrient-rich (YP) medium containing appropriate carbon source. YNB medium contained 0.67% yeast nitrogen base (YNB) with amino acids (Difco) supplemented with 2%

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glucose (YNBD), 2% glycerol (YNBG), 2% methanol (YNBM), or 0.5% oleic acid and 0.05% Tween 40 (YNBO). YP medium contained 1% yeast extract and 2% peptone supplemented with 2% glucose (YPD), 2% methanol (YPM), 2% glycerol (YPG), or 0.5% oleic acid and 0.05% Tween 40 (YPO). Cells were first cultured in YPD overnight in shake flasks, and the late log phase cultures were then inoculated into minimal or nutrient-rich medium containing non-fermentable carbon sources and cultured for different periods of time.

2.3. Generation of recombinant Trm1p

The gene encoding 220 N-terminal amino acids of Trm1p including the putative zinc finger domain, was obtained by PCR amplification of *P. pastoris* genomic DNA using the primer pair 5′-CCGCTCGAGATGCCTCCTAAACATCGG-3′ and 5′-CGGGGTACCCT ATTCTATGGAACCATCAATAGAAGC-3′ and cloned into XhoI and KpnI sites of pRSETA vector (Invitrogen, USA) using standard molecular biology protocols. The restriction sites in the primers were underlined. The recombinant plasmid was transformed into *E. coli* BL21 pLys-S and cells were grown at 37 °C until an A_{600} of 0.8 was reached. Expression of the recombinant protein was induced for 3 h by the addition of 1.0 mM IPTG. Cells were harvested and resuspended in buffer A (50 mM Tris–HCl [pH 8.0], 250 mM NaCl, 10% glycerol and 10 mM β-Mercaptoethanol) containing 10 mM imidazole. Cells were lysed by sonication and the lysate was centrifuged at 10,000*g* at 4 °C for 30 min. The supernatant was passed through Ni^{2+}-NTA Agarose column (Qiagen, USA) equilibrated with buffer A. The column was washed with buffer A containing 50 mM imidazole and the recombinant protein was eluted with buffer A containing 100 and 300 mM imidazole. The 300 mM elute fractions was pooled and stored at −80 °C. Western blot analysis of the purified protein was done with anti-histidine tag antibodies (Bangalore Genei, Bangalore, India).

2.4. Construction of P. pastoris ΔTrm1 strain

ΔTrm1 strain was generated by replacing the *TRM1* coding region with a zeocin expression cassette. The *TRM1* deletion construct consisted of zeocin expression cassette flanked by 1 kb *TRM1* promoter and 1 kb of 3′ untranslated region. The construct was transformed into *P. pastoris* by electroporation and the colonies were screened for *TRM* deletion by PCR of genomic DNA using appropriate primers.

2.5. Generation of P. pastoris expressing myc-tagged Trm1p ($Trm1p^{myc}$)

A 3.96 kb DNA comprising of −993 bp of *TRM1* promoter and 2967 bp of *TRM1* coding region was amplified from *P. pastoris* genomic DNA using the primer pair 5′ CGGGGTACCCCTTGAGTTTT TTCTCCGTTTCCTC 3′ (−993 nt to −696 nt) and 5′ ATAAGAATGC GGCCGCTACTGTCAAAATTTATTGTATCTGGCGC 3′ (2941 nt to 2967 nt) by PCR. The KpnI and NotI sites in the primers are underlined. The DNA was cloned at the KpnI and NotI sites of pGAPZA vector, in-frame with the vector-encoded myc tag. The recombinant plasmid was linearized with AvrII and transformed into *P. pastoris GS115* strain. Recombinants expressing myc-tagged Trm1p ($Trm1p^{myc}$) were selected by Western blotting using anti-myc antibodies.

2.6. Total RNA isolation and quantification

Total RNA was isolated from yeast cells by the hot phenol method [19]. Briefly, yeast cells grown in YPD medium were harvested by centrifuging at 1500*g* for 10 min at room temperature. The cells were resuspended in buffer B (50 mM sodium acetate [pH 5.3], 10 mM EDTA). To this, SDS was added to a final concentration of 1% and vortexed for 20 s. Next, equal volume of phenol saturated with buffer B was added and again vortexed for 20 s followed by incubation at 65 °C for 5 min. The tubes were placed in liquid nitrogen for 1 min and then centrifuged at 12,000 rpm for 10 min at 4 °C. The aqueous phase was removed carefully and extracted with phenol:chloroform (1:1). The upper aqueous phase was separated and sodium acetate (pH 5.3) was added to a final concentration of 300 mM followed by 2 volumes of ice-cold ethanol. The contents were mixed, frozen in liquid nitrogen and centrifuged at 12,000 rpm for 15 min at 4 °C. The RNA pellet was washed with 80% ethanol, air dried and dissolved in water. RNA was quantified by measuring absorbance at 260 nm, electrophoresed on 1.2 formaldehyde agarose gels and northern blotting was carried out as described [20]. Real-time PCR analysis was performed using iQ SYBR Green Super Mix and a iQ5multi-Color Real-time PCR thermal cycler (iCycler; Bio-Rad). Levels of mRNA expression in *Δmxr1 and Δtrm1* cells relative to *GS115* were normalized to 18S rRNA. 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, was used to analyze the data.

2.7. Synthesis of oligonucleotides and preparation of radiolabeled probes

All oligonucleotides were purchased from Sigma Aldrich Chemicals (Bangalore, India). Sense and antisense oligonucleotides were annealed by heating at 75 °C for 15 min in presence of 10 mM Tris–HCl [pH 7.5] and 100 mM NaCl followed by slow cooling to room temperature. ^{32}P-labeled DNA probes were generated by radio labeling the 5′ ends of double-stranded oligonucleotides with T4 polynucleotide kinase and [γ-^{32}P]ATP using standard molecular biology protocols [20]. Unincorporated radionucleotides were removed by Sephadex G-50 chromatography.

2.8. Electrophoretic mobility shift assay (EMSA)

Purified recombinant Trm1p-N or Mxr1pN (30 nM) was incubated with radiolabeled DNA probes (20,000 cpm, ∼0.2–0.5 nM) in a 20 μl reaction containing buffer A (50 mM Tris–HCl [pH 8.0], 50 mM NaCl, 1 mM DTT, 0.05% NP-40, 100 ng poly(dI:dC) and 6% glycerol for 30 min at 4 °C. The reaction mixture was electrophoresed at 4 °C on a 5% non-denaturating polyacrylamide gel in a buffer containing 7 mM Tris-HCl [pH 7.4], 3 mM boric acid and 1 mM EDTA. The gels were dried and autoradiographed.

2.9. Immunofluorescence

P. pastoris cells were first grown in YPD up to mid-logarithmic phase and then shifted to YPM or YNBM. After 16 h of growth, cells were treated with 1% formaldehyde for 1 h and spheroplasts were prepared by treating the cells with zymolase (5 mg/ml) in a buffer containing 50 mM Tris–HCl [pH 7.5], 10 mM $MgCl_2$, 1 mM DTT and 1 M sorbitol for 1 h at 37 °C. Cells were centrifuged at 5000 rpm for 10 min in a microfuge (eppendorf) at room temperature, resuspended in phosphate buffered saline (PBS), spread evenly onto glass cover slips and air dried. Cover slips were incubated in blocking buffer (PBS containing 0.05% Triton X-100 and 5% bovine serum albumin) containing mouse anti-myc antibody (1:50 dilution), washed with PBS and then incubated with TRITC-conjugated anti-mouse IgG (1:100 dilution) (Santacruz). Cover slips were washed with PBS and treated with 1 μg/ml of DAPI (Sigma–Aldrich) for 5 min. After washing with PBS, cover slips were air-

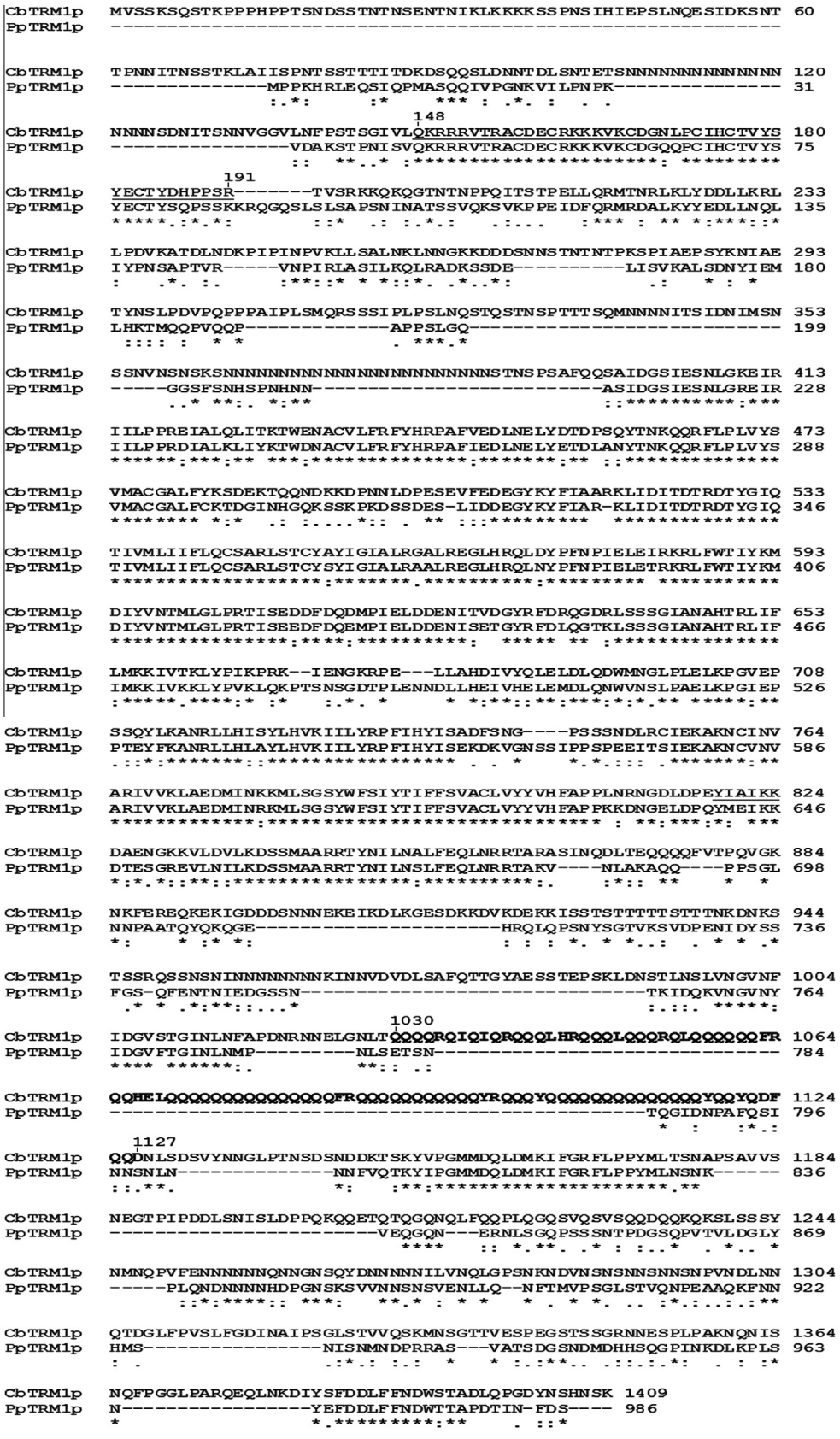

Fig. 1. Alignment of amino acid sequences of CbTrm1p and PpTrm1p. The zinc finger domain between amino acids 148 and 191 in CbTrm1p is underlined. The glutamine-rich domain in CbTrm1p between amino acids 1030 and 1127 is shown in bold face.

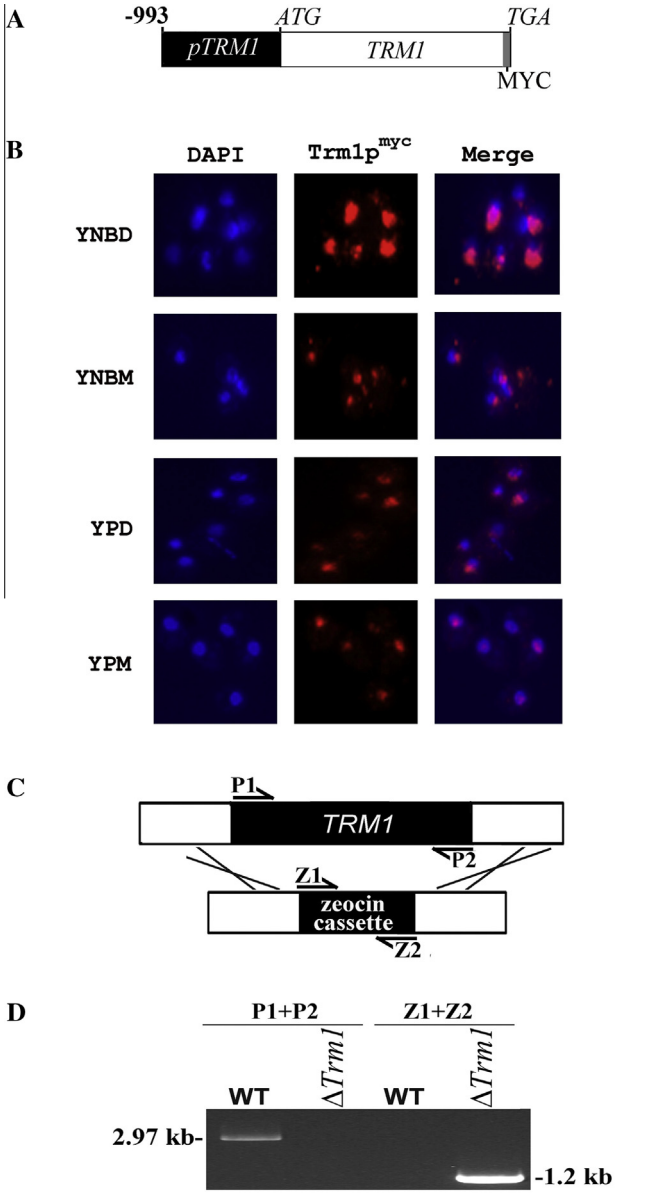

Fig. 2. Subcellular localization of PpTrm1p and characterization of *P. pastoris ΔTrm1* strain. (A) Schematic representation of the gene encoding PpTrm1p^{myc}. (B) Nuclear localization of PpTrm1p^{myc} in cells cultured in YNBD, YNBM, YPD or YPM using mouse anti-myc antibodies and TRITC-conjugated goat anti-mouse antibodies. Nuclei are stained with DAPI. (C) Schematic representation of the strategy for generating *ΔTrm1* strain. (D) Confirmation of *P. pastoris ΔTrm1* strain by PCR amplification of genomic DNA using appropriate primers as indicated. (E)–(G) Comparison of growth of *GS115*, *ΔMxr1* and *ΔTrm1* in YNBM (E), YPM (F) and YPG, YPO or YPA (G).

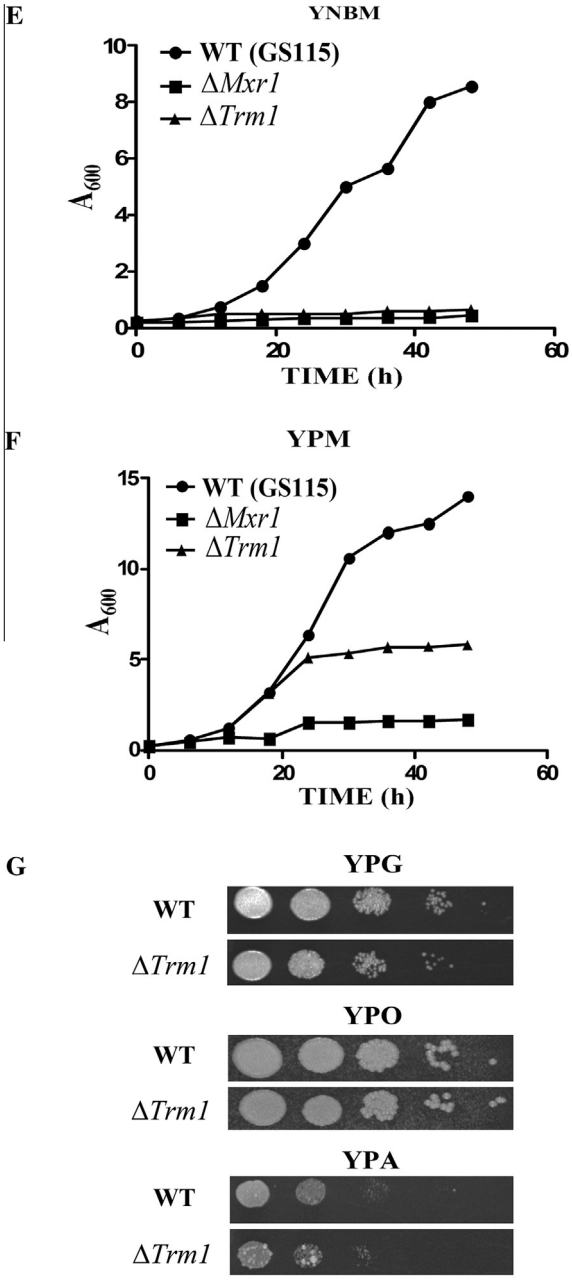

Fig. 2 (*continued*)

dried and cells were visualized in a fluorescent microscope (Leica) using appropriate filters.

3. Results

3.1. Characterization of P. pastoris Trm1p

Trm1p is a key regulator of methanol metabolism in *C. boidinii* [17]. In this study, we examined the role of Trm1p in the transcriptional regulation of mut pathway in *P. pastoris*. *P. pastoris* Trm1p (PpTrm1p) shares 58% amino acid sequence identity with *C. boidinii* Trm1p (CbTrm1p) (Fig. 1). Their zinc finger domains share 86% amino acid sequence identity. Interestingly, a glutamine-rich domain present in CbTrm1p between 1030 and 1117 amino acids is absent in PpTrm1p (Fig. 1). To study the sub-cellular localization of PpTrm1p, *TRM1* along with 1.0 kb of its own promoter was cloned into pGAPZA vector in-frame with the vector-encoded myc epitope was expressed in *P. pastoris GS115* strain. Subcellular localization of myc epitope-tagged PpTrm1p (Trm1p^{myc}) was examined by immunofluorescence using anti-myc antibodies. The results indicate that Trm1p^{myc} is localized to the nucleus of cells cultured in YNBD, YNBM, YPD or YPM (Fig. 2A and B).

3.2. Trm1p is a key regulator of methanol metabolism in P. pastoris

To examine whether Trm1p is essential for methanol metabolism, *P. pastoris ΔTrm1* strain in which *TRM1* coding region was replaced by a zeocin expression cassette was generated (Fig. 2C). Deletion of *TRM1* was confirmed by PCR amplification of genomic

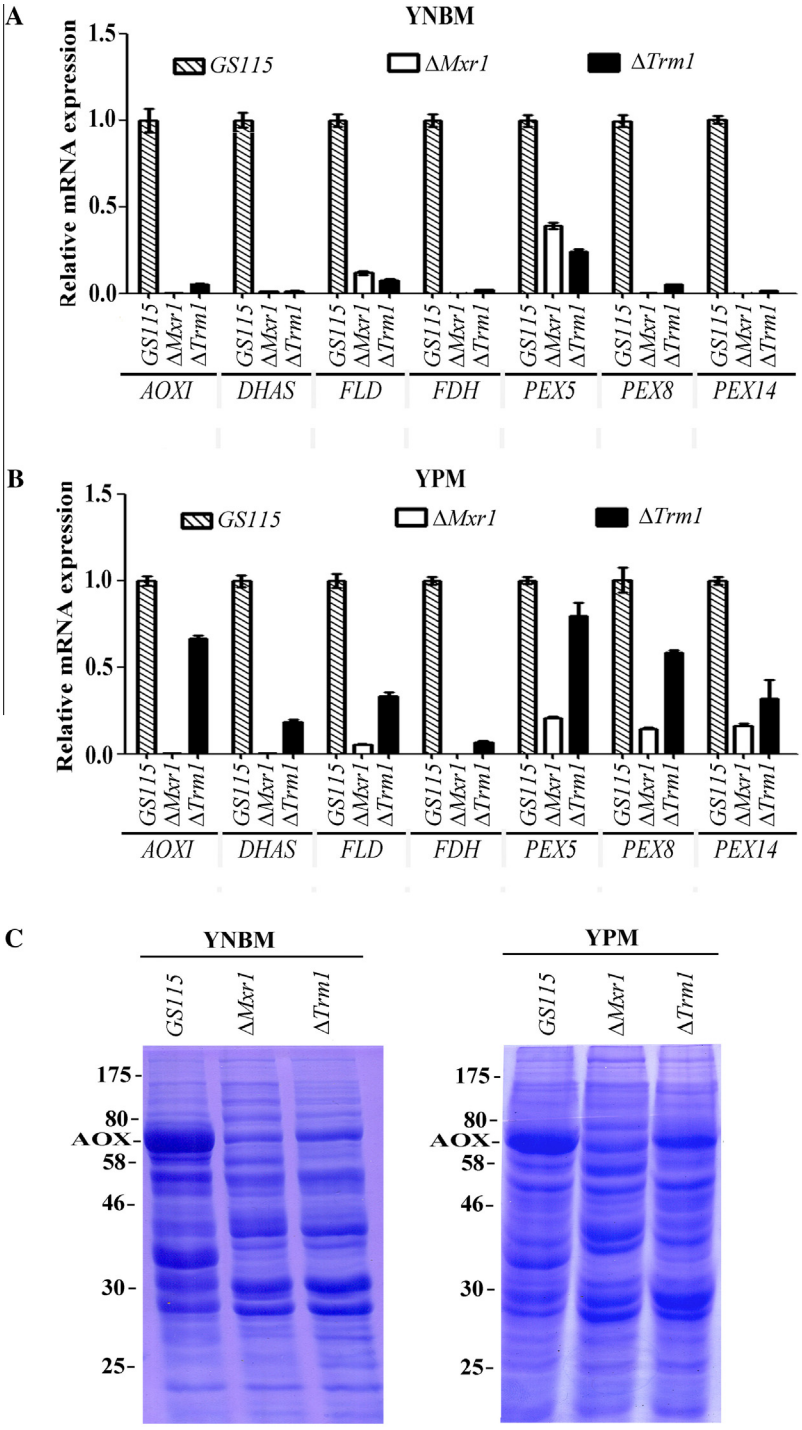

Fig. 3. Analysis of expression of genes of mut pathway. Quantification of expression of genes of mut pathway by Q-PCR analysis of RNA isolated from *P. pastoris* WT, *ΔMXR1* and *ΔTRM1* cultured in YNBM (A) or YPM (B). The histograms represent the average of the mean of three independent quantitative real-time PCR reactions, done in triplicate, ±standard deviation. mRNA expression was normalized to 18S rRNA. (C) Analysis of AOXI protein levels by SDS–PAGE of lysates prepared from *P. pastoris* cells cultured in YNBM or YPM. Gels were stained with Coomassie brilliant blue R.

DNA using appropriate primers (Fig. 2D). When cultured in YNBM or YPM, growth of *ΔTrm1* was severely impaired (Fig. 2E and F). Growth retardation of *ΔTrm1* was more severe in cells cultured in YNBM than YPM (Fig. 2E and F). No growth defect was observed when *ΔTrm1* was cultured on other nonfermentable carbon sources such as glycerol, oleic acid or acetate (Fig. 2G). Methanol-inducible expression of *AOXI*, *DHAS*, *FDH* encoding formate dehydrogenase, *FLD* encoding formaldehyde dehydrogenase, *PEX8*, *PEX5* and *PEX14* encoding peroxins 8, 5 and 14 respectively was severely affected in *ΔTrm1* cultured in YNBM (Fig. 3A) as well as YPM (Fig. 3B). AOX level is reduced in *ΔTrm1* cultured in YNBM as well as YPM (Fig. 3C). Interestingly, impaired expression of genes of mut pathway is more severe in *ΔTrm1* grown in YNBM than YPM (Fig. 3).

3.3. Analysis of DNA binding activity of Trm1p

To study the DNA binding specificity of PpTrm1p, the gene encoding 220 N-terminal amino acids of PpTrm1p, including the zinc finger domain, was expressed as his-tagged protein in *E. coli*

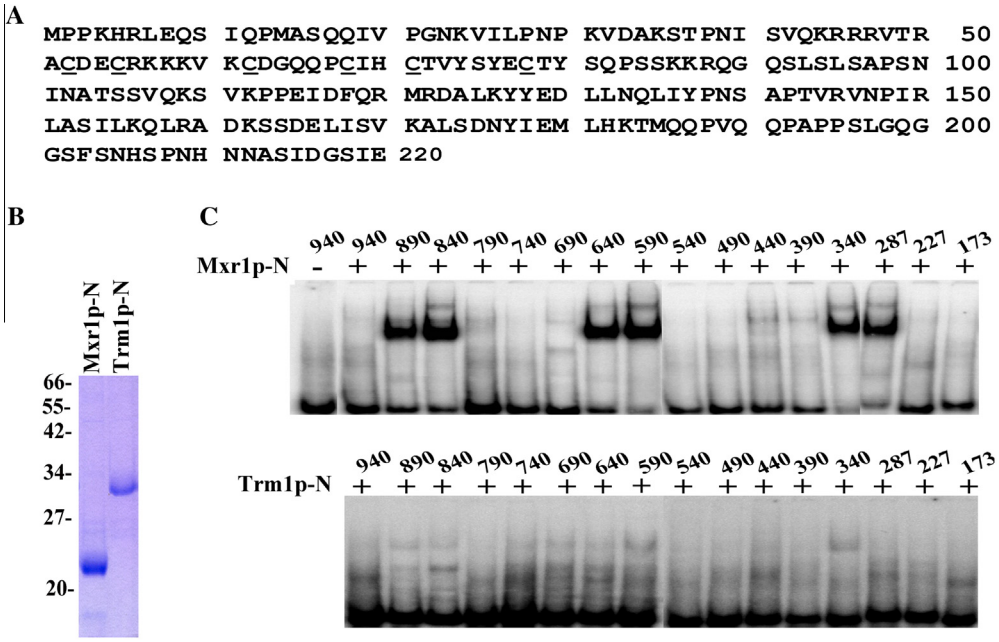

Fig. 4. Analysis of DNA binding activity of Trm1p-N and Mxr1p-N. (A) Amino acid sequence of Trm1p-N. (B) SDS–PAGE analysis of his-tagged Mxr1p-N and Trm1p-N after purification by Ni^{2+}-NTA affinity chromatography. (C) Analysis of binding of Mxr1p-N and Trm1p-N to ^{32}P-labeled *AOXI* promoter sequences by EMSA. Mxr1p-N and *AOXI* promoter sequences used in EMSA have been described [10].

and purified by Ni^{2+}-NTA affinity chromatography. The protein was named Trm1p-N and its ability to bind to *P. pastoris AOXI* promoter sequences was examined by EMSA. Recombinant Mxr1p comprising of 150 N-terminal amino acids (Mxr1p-N), which was earlier shown to bind to specific *AOXI* promoter sequences [10–12] was used as a positive control. The results indicate that Trm1p-N does not bind to *AOXI* promoter sequences under conditions recombinant Mxr1p-N binds specifically to six different *AOXI* promoter regions (Fig. 4). The weak DNA binding of Trm1p-N to certain *AOXI* promoter sequences was not reproducible.

4. Discussion

In *C. boidinii*, Trm1p and Trm2p are essential for the methanol-inducible expression of genes of the mut pathway [17,18]. The Trm2p homologue is known as Mxr1p in *P. pastoris*. Mxr1p is essential for the activation of genes of mut pathway [9,18]. The role of Trm1p in the transcriptional regulation of mut pathway in *P. pastoris* is not known. In this study, we demonstrate that Trm1p is essential for the transcriptional activation of genes of mut pathway, in addition to Mxr1p. Thus, two transcriptional activators are essential for the methanol-inducible expression of genes of mut pathway in *P. pastoris* as well as *C. boidinii*. An interesting observation made in this study is the fact that the effect of deletion of *TRM1* on the expression of genes of mut pathway is more pronounced in cells cultured in YNBM than those cultured in YPM. A similar but opposing effect was observed in case of Rop1p, which represses the expression of genes of mut pathway only in cells cultured in YPM but not YPM [12]. Unlike Rop1p, which is cytosolic in cells cultured in YNBM and nuclear in cells cultured in YPM [12], Trm1p localizes to the nucleus of cells cultured in YNBM as well as YPM. Taken together, our results indicate that media components other than methanol modulate the function of transcription factors such as Rop1p and Trm1p.

A large number of zinc finger proteins have been characterized in fungi [21] of which the *Saccharomyces cerevisiae* transcription factor Gal4p, is arguably the most well studied [22,23]. In case of Gal4p, which also contains a $Zn(II)_2Cys_6$-type zinc finger, 74 N-terminal amino acids comprising the zinc finger is sufficient to bind DNA [22] and this property was exploited for the development of the yeast two-hybrid system [23]. However, unlike Gal4p, PpTrm1p-N comprising 220 N-terminal amino acids failed to bind to *AOXI* promoter sequences *in vitro*. A similar observation was made in case of CbTrm1p as well [17]. CbTrm1p binds to five methanol-inducible promoters of cells grown in methanol *in vivo* as evident from chromatin immunoprecipitation assays, but fails to bind to methanol-inducible promoter sequences *in vitro* [17]. While Trm1p is a key regulator of methanol metabolism in *P. pastoris*, the exact mechanism by which Trm1p and Mxr1p (Trm1p and Trm2p in case of *C. boidinii*) co-ordinately regulate transcription is a topic of future study.

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