Catabolite repression of phosphoenolpyruvate carboxykinase by a zinc finger protein under biotin- and pyruvate carboxylase-deficient conditions in *Pichia pastoris*

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We have identified a methanol- and biotin-starvation-inducible zinc finger protein named ROP [repressor of phosphoenolpyruvate carboxykinase (PEPCK)] in the methylotrophic yeast *Pichia pastoris*. When *P. pastoris* strain GS115 (wild-type, WT) is cultured in biotin-deficient, glucose-ammonium (Bio[−]) medium, growth is suppressed due to the inhibition of anaplerotic synthesis of oxaloacetate, catalysed by the biotin-dependent enzyme pyruvate carboxylase (PC). Deletion of *ROP* results in a strain (Δ ROP) that can grow under biotin-deficient conditions due to derepression of a biotin- and PC-independent pathway of anaplerotic synthesis of oxaloacetate. Northern analysis as well as microarray expression profiling of RNA isolated from WT and Δ ROP strains cultured in Bio[−] medium indicate that expression of the phosphoenolpyruvate carboxykinase gene (*PEPCK*) is induced in Δ ROP during biotin- or PC-deficiency even under glucose-abundant conditions. There is an excellent correlation between *PEPCK* expression and growth of Δ ROP in Bio[−] medium, suggesting that ROP-mediated regulation of *PEPCK* may have a crucial role in the biotin- and PC-independent growth of the Δ ROP strain. To our knowledge, ROP is the first example of a zinc finger transcription factor involved in the catabolite repression of *PEPCK* in yeast cells cultured under biotin- or PC-deficient and glucose-abundant conditions.

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INTRODUCTION

Biotin, a water-soluble vitamin (vitamin H) is an essential nutrient for the growth of several laboratory strains of *Saccharomyces cerevisiae* as well as the methylotrophic yeast *Pichia pastoris*. It functions as the prosthetic group of several carboxylases involved in anaplerosis, fatty acid biosynthesis, gluconeogenesis and amino acid metabolism. In *S. cerevisiae*, biotin is essential for the catalytic activity of pyruvate carboxylase (PC), acetyl-CoA carboxylase, urea amidohydrolase and mitochondrial Hfa1p (Pirner & Stolz, 2006). Arc1p, a protein involved in cytosolic confinement of amino acyl tRNA synthetases, is also biotinylated (Kim *et al.*, 2004). Many laboratory strains of *S. cerevisiae* such as S288c possess only a partial biotin biosynthetic pathway and therefore cannot synthesize biotin *de novo*. However, biotin-prototrophic strains of *S. cerevisiae* have been reported (Phalip *et al.*, 1999; Wu *et al.*, 2005). *P. pastoris*

strain GS115 (wild-type, WT) does not possess a *de novo* biotin biosynthetic pathway. However, expression of four *S. cerevisiae* genes involved in biotin biosynthesis resulted in a *P. pastoris* strain capable of *de novo* biotin synthesis (Gasser *et al.*, 2010).

In general, yeast cells cannot grow in a biotin-deficient medium (Bio[−]) containing glucose and ammonium as sole sources of carbon and nitrogen respectively. Growth inhibition is primarily due to the non-replenishment of the citric acid cycle intermediate oxaloacetate (OAA) by the biotin-dependent enzyme PC. When such media are supplemented with aspartate, growth is restored due to replenishment of OAA via transamination of α -ketoglutarate (Zelle *et al.*, 2010; Blázquez *et al.*, 1995). OAA can be synthesized in a PC- and biotin-independent manner via the glyoxylate cycle when yeast cells are cultured in media containing non-fermentative carbon sources such as acetate. However, enzymes of the glyoxylate cycle are repressed under glucose-abundant conditions (Blázquez *et al.*, 1995). In *S. cerevisiae*, strains carrying mutations in the genes encoding PC and pyruvate kinase, phosphoenolpyruvate carboxykinase (PEPCK), which normally has decarboxylating and gluconeogenic functions, can replace the anaplerotic function of PC in glucose-grown cultures (Zelle *et al.*, 2010). While PC-deficient yeast cells cannot grow in a medium

Abbreviations: ICL, isocitrate lyase; MS, malate synthase; Mut, methanol utilization; OAA, oxaloacetate; PC, pyruvate carboxylase; PEP, phosphoenolpyruvate; PEPCK, phosphoenolpyruvate carboxykinase.

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Three supplementary figures are available with the online version of this paper.

containing glucose and ammonium as the sole sources of carbon and nitrogen respectively, certain mutations termed *BPC* (bypass of pyruvate carboxylase) enable them to grow under these conditions (Blázquez *et al.*, 1995). *BPC* mutants synthesize OAA by a PC-independent mechanism involving either derepression of enzymes of the glyoxylate pathway or by other mechanisms that bypass PC function (Blázquez *et al.*, 1995). However, the transcription factors responsible for the *BPC* phenotype are not known.

Mxr1p is a zinc finger protein that plays a key role in the regulation of genes of the methanol utilization (Mut) pathway as well as those encoding peroxisomal enzymes in *P. pastoris* (Lin-Cereghino *et al.*, 2006). Mxr1p regulates the expression of target genes by binding to MXR response elements (MXREs) comprising of a core 5'-CYCCNY-3' motif (Kranthi *et al.*, 2009, 2010). The *P. pastoris* genome encodes a large number of zinc finger proteins, many of which are still not well characterized (De Schutter *et al.*, 2009). In this study, we have characterized a novel methanol-inducible zinc finger protein named ROP which has a DNA-binding domain similar to that of Mxr1p but with a different biological function. In addition to methanol, ROP expression is also induced in *P. pastoris* cells by biotin deficiency and ROP regulates the expression of several genes including that encoding the gluconeogenic enzyme PEPCK.

METHODS

Media, yeast strains and yeast culture techniques. *P. pastoris* strain GS115 (*his4*) was cultured at 30 °C in shake flasks containing YPD medium (1 % yeast extract, 2 % peptone and 2 % glucose) or YNB medium (0.67 % yeast nitrogen base without amino acids) supplemented with 50 µg histidine ml⁻¹ and either 2 % glucose (YNBD), 2 % (v/v) glycerol (YNBG), 2 % methanol (YNBM), or 0.5 % oleic acid and 0.05 % Tween 40 (YNBO). For studying the effect of biotin, *P. pastoris* cells were grown in a synthetic mineral (SM) medium containing (Bio⁺) or lacking (Bio⁻) biotin. One litre of SM contained 20 g glucose, 4 mg L-histidine, 200 ml 5 × salts, 10 ml 100 × vitamin mixture and 10 ml 100 × trace element mixture. The 5 × salt solution was prepared by dissolving 5 g (NH₄)₂HPO₄, 1.12 g MgSO₄, 1.23 g KCl and 125 mg NaCl in 100 ml water. A 100 ml solution of 100 × vitamin mixture consisted of 1 g thiamine hydrochloride and 3 mg biotin. A 100 ml preparation of 100 × trace element mixture contained 1 mg H₃BO₃, 1 mg NiSO₄, 1 mg KI, 1 mg Na₂MoO₄·2H₂O, 1 mg CoCl₂, 0.5 g Fe(NH₄)(SO₄)₂, 40 mg CuSO₄, 150 mg ZnSO₄·7H₂O, 240 mg MnSO₄·H₂O and 0.5 g EDTA. During the preparation of Bio⁻ medium, biotin was omitted and avidin (23 µg ml⁻¹) was added to remove trace amounts of biotin present in the medium. In some experiments, phenyl acetic acid (2.5 mM) was added to Bio⁺ media to inhibit PC.

P. pastoris was transformed with DNA by electroporation (Gene Pulser Xcell Electroporation System, Bio-Rad) as per the manufacturer's instructions. Zeocin-resistant colonies were selected on YPD plates containing zeocin (100 µg ml⁻¹). For the study of ROP expression in *P. pastoris* cultured with different carbon sources, cells were first grown overnight in YPD medium to stationary phase. An aliquot of the stationary culture was used to inoculate YNBD, YNBM, YNMG or YNBO medium at OD₆₀₀ ~0.5 and cells were grown for 12–24 h.

Nucleic acid isolation, Northern and Southern blot analysis. Total RNA was isolated from *P. pastoris* cells by the hot-phenol

method (Schmitt *et al.*, 1990). Electrophoresis of RNA on formaldehyde-agarose gels and Northern analysis was carried out essentially as described by Maniatis *et al.* (1982). Isolation of *P. pastoris* genomic DNA, random-primed labelling of cDNAs with Klenow polymerase and [α -³²P] dCTP, and Southern blotting, were carried out using standard protocols (Maniatis *et al.*, 1982). The cDNA probes used for Northern and Southern analysis were obtained by PCR amplification of *P. pastoris* genomic DNA using the following gene-specific primers: *AOXI* (alcohol oxidase I), 5'-atggctatccccgaagatttg-3' and 5'-aagcct-caagaagctctggca-3'; *FLDI* (formaldehyde dehydrogenase I), 5'-atgtc-taccgaaggtcaaatcatc-3' and 5'-ggagctctgtcttgactttaacc-3'; *DHAS* (dihydroxyacetone synthase), 5'-atggctagaattccaaagcagtagc-3' and 5'-ccaacaatagcatagatagttgtc-3'; *PEX8* (peroxin 8), 5'-atgtatagattgggtagcagggag-3' and 5'-acgtcatctctcttgatgtgag-3'; *PEX14* (peroxin 14), 5'-atgtccagtatacgtgaagaaat-3' and 5'-atgactttataagcccaacgaac-3'; *BI02* (biotin synthase), 5'-atgaacagacagctgtgtgagcag-3' and 5'-ctttctgtgtctaacagttctgtatg-3'; *BPL1* (biotin-apoprotein ligase), 5'-atgcaactccgaacctgtagac-3' and 5'-acagctcaccacatccaggtagc-3'; *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), 5'-atggctatcatctgctgttataac-3' and 5'-gaaagt-tctgttgacaacctggc-3'; *PEPCK* (phosphoenolpyruvate carboxykinase), 5'-atggctctactgtatagattac-3' and 5'-ttctcagtagggcgatcaacatg-3'; *ICL* (isocitrate lyase), 5'-atgtcaggaccttacactaagattgac-3' and 5'-gaagtcagtg-taaggcaacttagctc-3'; *MS* (malate synthase), 5'-atgacccccagcaacgtata-taac-3' and 5'-taactgtccgtaataatattagacc-3'; *ROP*, 5'-cgcgatccatgtc-tgctcatctcc-3' and 5'-cccaagctttcaaccaacggttggtggcag-3'.

Disruption of ROP and generation of the Δ ROP strain. The Δ ROP strain was generated by replacing 2685 bp of ROP coding region (+1 to +2685 bp) with a zeocin expression cassette. The ROP knockout construct consisting of a zeocin expression cassette flanked by 930 bp of ROP promoter at the 5' end and 930 bp of ROP 3' untranslated region at the 3' end was generated by four different PCRs using *P. pastoris* genomic DNA and pGAPZA vector (Invitrogen) as templates as well as a series of overlapping and non-overlapping primers. To begin with, 930 bp of ROP promoter (from position -930 to -1) was amplified from *P. pastoris* genomic DNA using primers 5'-gctaagttagtcattgattaccgcttggaac-3' (1F: positions -930 to -898 of ROP promoter) and 5'-AGCTATGGTGTGTGGGGATCCGCAtcttggtgtt-ttcacaacgcaaa-3' (1R: nucleotides 986–962 of pGAPZA (upper case); nucleotides -1 to -25 of ROP promoter (lower case)). In another PCR, a 1.17 kb region of the pGAPZA vector (between positions 1419 and 2591) comprising the *TEF1* and *EM7* promoters, zeocin resistance gene (*ShBle*) and *CYC1* transcription termination region was amplified using the primer pair 5'-tttgcgttggaacaaacccaagaTGCGGATCCCCAC-ACACCATAGCT-3' (2F: positions -25 to -1 of the ROP promoter (lower case); positions 962–986 of pGAPZA (upper case)) and 5'-gtaaatgcatagtcattattttaccTGCTCATATGTTGGTCTCCAGCTTG-3' (2R: positions 2710 to 2686 of ROP 3' untranslated region (lower case); nucleotides 2160–2136 of pGAPZA (upper case)). In the third PCR, 930 bp of the 3' untranslated region of ROP was amplified from *P. pastoris* genomic DNA using the primer pair 5'-CAAGCTGG-AGACGAACATGTGAGCAGgtaaataatgactatgattac-3' (3F: nucleotides 2136–2160 of pGAPZA (upper case); nucleotides 2686–2710 of ROP (lower case)) and 5'-aatgtgtacgtgagtgcttcagaagaag-3' (3R: nucleotides 3616–3586 of ROP). Finally, the three PCR products containing overlapping regions were pooled and used as templates in a PCR along with primers 1F and 3R. The final 3.06 kb PCR product thus obtained was used to transform *P. pastoris* strain GS115 to generate the zeocin-resistant Δ ROP strain.

Gene expression profiling using *P. pastoris* microarray. A custom *P. pastoris* 8 × 15K microarray (Amadid no: 025088) was designed by Genotypic Technology (<http://www.genotypic.co.in>) based on the annotated *P. pastoris* genome sequence (Taxonomy ID: 644223) available in GenBank. Total RNA was isolated from *P. pastoris* cells by the hot-phenol method (Schmitt *et al.*, 1990) and RNA integrity was assessed using an RNA 6000 Nano Lab Chip on the

Fig. 1. Comparative analysis of zinc finger domains of ROP, Mxr1p and Adr1p, and analysis of *ROP* expression in *P. pastoris*. (a) Schematic representation of ORFs of Adr1p, Mxr1p and ROP and alignment of their amino acid sequences encompassing the N-terminal DNA-binding domain (black boxes). Numbers indicate amino acid residues. The length of each ORF is indicated in parentheses. Amino acids of the two zinc fingers are underlined. (b) Comparison of the putative C_2H_2 zinc finger domain of ROP with those of Adr1p and Mxr1p. The core nucleotide sequences essential for the binding of Adr1p and Mxr1p to DNA are shown in parentheses (N, any nucleotide; R, C/T; Y, A/G). The percentage amino acid sequence identity amongst the zinc fingers is indicated. (c) Northern blot analysis of *Mxr1*, *ROP* and *AOX1*.

2100 Bioanalyser (Agilent) as per the manufacturer's protocol. Total RNA purity was assessed by the NanoDrop ND-1000 UV-Vis Spectrophotometer (Nanodrop Technologies). Total RNA with $A_{260}:A_{280} > 1.8$ and $A_{260}:A_{230} \geq 1.3$ was used for microarray experiments. RNA was considered to be good for use in microarray when the rRNA 28S:18S ratios were ≥ 1.5 , with the rRNA contribution being 30% or more and the RNA integrity number (RIN) was ≥ 7.0 . RNA samples were labelled using the Agilent Quick-Amp labelling kit (p/n5190-0442). Five hundred nanograms of each of the control and test samples was incubated with reverse transcription mix at 40 °C and converted to double-stranded cDNA primed by oligo-dT with a T7 polymerase promoter. The cleaned-up double-stranded cDNAs were used as templates for complementary RNA (cRNA) generation. cRNA was generated by *in vitro* transcription and the dye Cy3 CTP (Agilent) was incorporated during this step. The cDNA synthesis and *in vitro* transcription steps were carried out at 40 °C. Labelled cRNA was cleaned up and quality was assessed for yields and specific activity. The labelled cRNA samples were

hybridized to the *P. pastoris* 8 × 15K microarray. Fragmentation of labelled cRNA and hybridization were done using the Gene Expression Hybridization kit from Agilent (Part Number 5188-5242). Hybridization was carried out in Agilent's Surehyb Chambers at 65 °C for 16 h. The hybridized slides were washed using Agilent Gene Expression wash buffers (Part No: 5188-5327) and scanned using the Agilent Microarray Scanner G2505C at 5 µm resolution. Data extraction from images was done using Feature Extraction software version 10.5.1.1 from Agilent. Feature-extracted data were analysed using GeneSpring GX Version 11 software from Agilent. Normalization of the data was done in GeneSpring GX using the percentile shift; percentile shift normalization is a global normalization, where the locations of all the spot intensities in an array are adjusted. This normalization takes each column in an experiment independently and computes the percentile of the expression values for this array, across all spots (where n has a range from 0 to 100 and $n=75$ is the median). It subtracts this value from the expression value of each entity and normalizes these to specific samples. Genes whose

expression was different by twofold and above were identified. Differentially regulated genes were clustered using hierarchical clustering to identify significant gene expression patterns.

RESULTS

Analysis of *ROP* expression and identification of *ROP*-regulated genes during methanol metabolism

We recently demonstrated that Mxr1p, a master regulator of the Mut pathway in *P. pastoris*, regulates gene expression by binding to promoter sequences containing 5'-CYCCNY-3' motif (Kranthi *et al.*, 2009, 2010). The DNA-binding domain of Mxr1p shares 55 % amino acid sequence identity with that of the *S. cerevisiae* zinc finger transcription factor Adr1p (Lin-Cereghino *et al.*, 2006, Fig. 1), whose recognition sequence contains 5'-CYCCNR-3' rather than 5'-CYCCNY-3' motifs (Kranthi *et al.*, 2009). A BLAST search of the *P. pastoris* genome sequence database with the amino acid sequence of Mxr1p led to the identification of a gene encoding a putative zinc finger protein (GenBank accession number CAY70604/XM_002492738.1). This protein, referred to as ROP (for repressor of *PEPCK*) in this study, was characterized in detail. The deduced amino acid sequence of ROP contains a putative C₂H₂ zinc finger domain, which shares 58 % and 55 % amino acid sequence identity with the zinc finger domains of Mxr1p and *S. cerevisiae* Adr1p respectively (Fig. 1a, b). While *Mxr1* is constitutively expressed in *P. pastoris* cells cultured with different carbon sources (Lin-Cereghino *et al.*, 2006, Fig. 1c), *ROP* expression was induced in *P. pastoris* cells cultured in a medium containing methanol (YNBM) but not glucose (YNBD), glycerol (YNBG) or oleic acid (YNBO) as the sole source of carbon (Fig. 1c). Methanol-inducible expression of *ROP* as well as the significant levels of homology between the DNA-binding domains of ROP and Mxr1p led us to examine whether ROP has a role in the regulation of genes of the methanol utilization (Mut) pathway.

As a first step, we generated a *P. pastoris* strain (Δ ROP) in which the entire ORF of *ROP* was replaced by a zeocin expression cassette (Fig. 2a). Deletion of the *ROP* coding region and loss of methanol-inducible expression of *ROP* were confirmed by Southern and Northern analysis respectively (Fig. 2b, c). Northern analysis of Mxr1p-regulated genes of the Mut pathway such as *AOXI* (alcohol oxidase I), *FLDI* (formaldehyde dehydrogenase I) and *DHAS* (dihydroxyacetone synthase) as well as peroxisomal genes such as *PEX8* and *PEX14* indicated that expression of these genes is not affected in Δ ROP (Fig. 2d). Furthermore, there was no significant difference in growth rates of WT (GS115) and Δ ROP strains cultured in YNBM medium (data not shown). To understand the physiological significance of methanol-inducible expression of ROP, transcripts which are expressed in WT and Δ ROP strains cultured in YNBM medium were hybridized to a *P. pastoris* microarray designed based on the *P. pastoris* genome

Fig. 2. Construction of Δ ROP and Northern analysis of genes involved in methanol metabolism. (a) Strategy for the generation of Δ ROP. The coding region of *ROP* was replaced by the zeocin expression cassette by homologous recombination (see Methods for details). Restriction enzymes used for confirmation of *ROP* deletion are indicated. The region corresponding to the DNA-binding domain is indicated by a grey box. (b) Southern analysis of *HincII*- and *BstXI*-digested genomic DNA isolated from WT and Δ ROP strains. Fragments of 2987 bp (*HincII*) and 2887 bp (*BstXI*) are generated in WT but not Δ ROP, confirming deletion of the *ROP* coding region. (c) Northern analysis of RNA isolated from WT and Δ ROP strains cultured in YNB medium containing glucose ('dextrose', D), glycerol (G), methanol (M) or oleic acid (O) as the sole source of carbon. The cDNA encoding the DNA-binding domain of ROP was radiolabelled and used to probe Southern and Northern blots. (d) Northern analysis of genes encoding enzymes of methanol metabolism and peroxins in WT and Δ ROP strains cultured in YNBM medium.

Fig. 3. Heat map of genes upregulated or downregulated in ΔROP cultured in YNB medium for 6 h or 24 h. Annotated genes which are upregulated at both 6 h and 12 h of growth are indicated by asterisks.

sequence data available in GenBank (De Schutter *et al.*, 2009). The results indicate that expression of several annotated as well as unannotated protein-coding genes is altered in ΔROP grown in YNB medium for 6 h or 24 h (Fig. 3). Expression of certain genes involved in biotin metabolism such as those encoding biotin synthase, vitamin H transporter and biotin–apoprotein ligase was increased in the ΔROP strain cultured in YNB medium for 6 h as well as 24 h (Fig. 3). Expression of genes of the Mut pathway was not affected in ΔROP , as observed by Northern analysis.

ROP function in cells cultured under glucose-abundant, biotin-deficient conditions

The activation of genes of biotin metabolism in the ΔROP strain cultured in YNB medium at both 6 h and 24 h led us to investigate whether ROP has a regulatory role in biotin metabolism. As a first step, we examined the growth of WT and ΔROP strains in biotin-sufficient (Bio⁺) or biotin-deficient (Bio⁻) medium containing glucose and ammonium as the sole source of carbon and nitrogen respectively. While the WT strain could grow only in Bio⁺

medium, ΔROP was able to grow in both Bio⁺ and Bio⁻ media (Fig. 4a). Interestingly, *ROP* expression was induced in WT cells grown in Bio⁻ medium (Fig. 4b). The biotin-independent growth of ΔROP is not due to *ROP*-mediated derepression of the *de novo* biotin biosynthetic pathway since genes encoding biotin synthase and biotin–apoprotein ligase were derepressed in the WT as well as the ΔROP strain cultured in Bio⁻ medium (Fig. 4c).

Catabolite repression of *PEPCK* in *P. pastoris* cultured under biotin- and PC-deficient, glucose-abundant conditions

Anaplerotic synthesis of OAA from phosphoenolpyruvate (PEP) is primarily catalysed by the biotin-dependent enzyme PC (Fig. 5a). It is known that aspartate can restore the growth of PC mutants in glucose-ammonium medium due to its conversion to OAA by transamination with

α -ketoglutarate (Zelle *et al.*, 2010; Blázquez *et al.*, 1995). When aspartate was added to Bio⁻ medium, growth of the WT strain was restored, indicating that the growth defect is primarily due to the deficiency of PC-mediated OAA synthesis (Fig. 5b). Furthermore, addition of phenylacetic acid (an inhibitor of PC) to Bio⁺ cultures completely inhibited the growth of WT, but not ΔROP (Fig. 5c). These results indicate that a PC-independent pathway of OAA synthesis (pyruvate carboxylase bypass) is responsible for the growth of ΔROP in Bio⁻ medium. Since enzymes of the

Fig. 4. Effect of biotin on *ROP* expression and growth of *P. pastoris*. (a) Growth of WT and ΔROP strains in Bio⁻ medium. Biotin was added after 24 h of growth as indicated by the arrow. (b) Northern analysis of *ROP* in WT and ΔROP cultured in Bio⁺ and Bio⁻ medium. (c) Northern analysis of the genes encoding biotin synthase (BS) and biotin–apoprotein ligase (BAL) in WT and ΔROP strains cultured in Bio⁺ and Bio⁻ medium.

Fig. 5. Effect of aspartate and phenylacetic acid on the growth of *P. pastoris*. (a) Schematic representation of biosynthesis of OAA and PEP. (b) Effect of aspartate on the growth of WT and ΔROP strains in Bio⁻ medium. (c) Effect of phenylacetic acid (PAA) (2.5 mM) on the growth of WT and ΔROP in Bio⁺ medium.

glyoxylate cycle such as isocitrate lyase (ICL) and malate synthase (MS) as well as *PEPCK* are known to substitute for PC and contribute to OAA synthesis under specific growth conditions (Zelle *et al.*, 2010; Blázquez *et al.*, 1995). We examined their expression in WT and Δ ROP strains. Northern analysis indicated that biotin deficiency abolishes catabolite repression of *ICL* and *MS* in the WT as well as in Δ ROP (Fig. 6a). However, catabolite repression of *PEPCK* in Bio⁻ cultures was abolished only in Δ ROP but not in the WT strain (Fig. 6a). Furthermore, *PEPCK* was derepressed by phenylacetic acid in Bio⁺ cultures of Δ ROP but not the WT (Fig. 6b). Thus, inactivation of PC either by biotin deficiency or by PAA results in the derepression of *PEPCK* in Δ ROP but not the WT under glucose-abundant conditions. When methanol is the sole carbon source, derepression of *PEPCK* is observed in both the WT and Δ ROP (Fig. 6c). Thus, ROP is involved in the catabolite repression of *PEPCK* specifically in glucose-abundant, biotin- and PC-deficient cultures of *P. pastoris*.

The DNA-binding domain of ROP shows significant homology to those of Adr1p and Mxr1p (Fig. 1). We reported earlier that Adr1p and Mxr1p bind to promoter sequences containing 5'-CYCCNY-3' and 5'-CYCCNR-3' respectively (Kranthi *et al.*, 2009, 2010). In view of the high

degree of homology among the DNA-binding domains of ROP, Mxr1p and Adr1p (Fig. 1), it is likely that the DNA-binding specificity of ROP is similar to that of Adr1p or Mxr1p. To examine whether 5'-CYCCNY-3'/5'-CYCCNR-3' is present in the *PEPCK* promoter, we carried out an *in silico* analysis and identified potential ROP-binding motifs in the *PEPCK* promoter (see Supplementary Fig. S1, available with the online version of this paper). Interestingly, ROP is also involved in the regulation of a number of genes other than *PEPCK*, as is evident from the microarray expression profiling of RNA isolated from Bio⁻ cultures of *P. pastoris* (Supplementary Fig. S2). The results from the microarray were further validated by Northern analysis of selected genes (Supplementary Fig. S3). More studies are needed to understand the physiological significance of upregulation of these genes.

DISCUSSION

In this study, we have characterized a zinc finger protein named ROP whose expression is induced by methanol and by biotin and pyruvate carboxylase (PC) deficiency. Methanol-inducible expression of ROP results in the activation and repression of a number of genes including those involved in biotin metabolism. However, genes of the Mut pathway as well as peroxisomal genes which are regulated by Mxr1p are not the targets of ROP. The activation of genes of biotin metabolism such as biotin synthase and vitamin H transporter during methanol metabolism in the Δ ROP strain led us to investigate the role of ROP under biotin-deficient conditions. We demonstrate that ROP represses biotin-independent growth of *P. pastoris*. In glucose-abundant cultures, biotin deficiency results in the activation of ROP and repression of *PEPCK*. Comparison of expression profiles of WT and Δ ROP strains indicates that ROP regulates the expression of several genes under biotin-deficient conditions. Since biotin deficiency inactivates PC, a key enzyme involved in the anaplerotic synthesis of OAA, we focused our attention on enzymes that are known to substitute for PC under certain growth conditions. We demonstrate that ROP has a key role in the derepression of *PEPCK* but not *ICL* and *MS* in biotin- and PC-deficient, glucose-abundant cultures of *P. pastoris*. The normal function of *PEPCK* is to catalyse the conversion of OAA to PEP. However, under certain conditions, *PEPCK* can catalyse the reverse reaction leading to the synthesis of OAA from PEP (Zelle *et al.*, 2010; Blázquez *et al.*, 1995). There is an excellent correlation between derepression of *PEPCK* and biotin- and PC-independent growth of Δ ROP. It is therefore tempting to speculate that *PEPCK*-catalysed OAA synthesis may have a key role in the biotin- and PC-independent growth of Δ ROP. It is pertinent to note that OAA can also be synthesized in a biotin- and PC-independent manner via the glyoxylate cycle through *ICL* and *MS*. However, derepression of *ICL* and *MS* does not restore growth of the WT strain in Bio⁻ medium. Thus, if OAA is synthesized via the glyoxylate cycle in Bio⁻ cultures

Fig. 6. Northern analysis of the genes encoding isocitrate lyase (*ICL*), malate synthase (*MS*) and *PEPCK* (*PEPCK*). (a) Expression of these genes in WT and Δ ROP strains cultured in Bio⁺ and Bio⁻ medium. (b) Effect of PAA on *PEPCK* expression in WT and Δ ROP cultured in Bio⁺ medium. (c) *PEPCK* expression in WT and Δ ROP strains cultured in YNB medium.

of *P. pastoris*, synthesis of PEP from OAA by PEPCK may be the growth-limiting reaction. Thus, either the gluconeogenic activity or the anaplerotic function of PEPCK may be responsible for the biotin- and PC-independent growth of Δ ROP. More studies are needed to distinguish between these possibilities. ROP appears to be the first example of a yeast transcription factor that is specifically involved in catabolite repression of PEPCK under biotin- and PC-deficient conditions.

The phenotype of Δ ROP described in this study is similar to that of BPC mutants of *S. cerevisiae* (Blázquez *et al.*, 1995). Both can grow in glucose-ammonium medium, despite PC deficiency. However, ICL is involved in overcoming PC deficiency in *S. cerevisiae* BPC mutants whereas PEPCK is involved in overcoming PC deficiency in Δ ROP. While the transcription factors involved in the BPC phenotype are not known, we have demonstrated that ROP is responsible for the biotin- and PC-independent growth phenotype of Δ ROP. Spontaneous mutants of PC-deficient *P. pastoris* strains, which can grow in glucose-ammonium medium, have been reported (Menéndez *et al.*, 1998) and it will be interesting to examine ROP expression in these mutants.

Although the physiological significance of ROP expression during methanol metabolism is not clear at present, we wish to discuss an unusual function of PC during methanol metabolism which may be responsible for the activation of ROP in methanol-grown cultures of *P. pastoris*. PC is required for the assembly and peroxisomal import of alcohol oxidase, the first enzyme involved in methanol metabolism in methylotrophic yeasts such as *P. pastoris* and *Hansenula polymorpha* (Ozimek *et al.*, 2003, 2007). PC mediates flavin adenine dinucleotide (FAD) binding to AOX in the cytosol and this reaction is essential for its subsequent octamerization and import into peroxisomes. The transcarboxylation domain of PC, which participates in the transfer of the carboxyl group of carboxylated biotin to pyruvate, is involved in AOX assembly (Ozimek *et al.*, 2003, 2007). Since AOX is the most abundant protein present in methanol-grown *P. pastoris* cells, it is possible that PC is primarily utilized for the assembly of AOX during methanol metabolism rather than for the synthesis of OAA. Thus, a decrease in the anaplerotic activity of PC, as observed in biotin- and PC-deficient (Bio^- ; PAA^+ , Bio^+) cultures may occur during methanol metabolism as well. Thus, deficiency in the catalytic activity of PC may serve as a signal for the activation of ROP in Bio^- as well as YNB media. It should however be noted that ROP does not regulate PEPCK expression during methanol metabolism and its exact function during methanol metabolism is not clear at present. A detailed analysis of the function of ROP-regulated genes during methanol metabolism will be reported separately.

In summary, ROP is a novel methanol- and biotin-starvation-inducible zinc finger protein that represses the growth of *P. pastoris* under biotin- and PC-deficient, glucose-abundant conditions. ROP is specifically involved

in catabolite repression of PEPCK and to our knowledge is the first yeast transcription factor that is involved in the catabolite repression of PEPCK under biotin- and PC-deficient conditions.

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