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CARBON Repression in PICHIA PASTORIS

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ABSTRACT: One of the popularly studied yeasts used for heterologous protein production is methylotrophic Pichia pastoris. This success is due to its robust methanol-inducible alcohol oxidase 1 (AOX1) promoter, and its ability to carry out post-translational changes and pathways leading to the secretion of recombinant products. This promoter depends strictly on the carbon source present in the medium; the methanol is the preferred one. The other enzymes required to use alternative carbon sources are synthesized at weak rates or not at all when methanol is present. Catabolite repression or carbon catabolite repression is called this phenomenon. In this review, we will collect all the information gathered in recent years about a variety of repressible catabolite systems in Pichia pastoris.

KEYWORDS*:* catabolite repression,carbon source,pichia pastoris

I. BACKGROUND

A. METHYLOTROPHIC YEASTS

Methylotrophic yeasts like Hansenula polymorpha (Pichia angusta), Pichia methanolica, Candida boidinii, and Komagataella pastoris are prominent hosts for the production of heterologous proteins[1, 2].these yeasts can metabolize methanol as the sole unique source of carbon and energy. A growing number of biotechnological applications and biopharmaceuticals have resulted in the favorable and most advantageous characteristics of this specie[3]. The most crucial fact of methylotrophic yeasts is that methanol highly induces most of the genes involved in the methanol utilization pathway, and the glucose represses them[4]. Promoters of genes imply in the MUT pathway, such as those coding alcohol oxidase (MOX, MOD1, AOD1 or AOX1) and dihydroxyacetone synthase (DHAS or DAS), have been used to express heterologous proteins[5]. Although enzymes and metabolic pathways involved in the MUT pathway are similar in different methylotrophic yeasts, the gene transcriptional regulatory profiles are different[6-8]. The capacity of P. pastoris to use methanol as the unique carbon source is a crucial aspect of its metabolism[9]. It can also be an excellent model organism for the investigation of methanol assimilation and peroxisomal proliferation.

B. METHANOL METABOLISM IN THE METHYLOTROPHIC YEASTS

By definition, methylotrophs are the microorganisms capable of using reduced form C1compounds as a sole carbon and energy source[10]. A variety of prokaryotes and eukaryotes can be used to grow C1 compounds, and methylotrophs have a variety of metabolic pathways to dissimile and assimilate C1 compounds[11]. C1compounds such as methanol, methane, formaldehyde, methylated sulfur, methylated amines compounds, and methyl halogenates are the main methylotrophs substrates[12]. While prokaryotic methylotrophs can use all of these substrates as sole sources of carbon and energy, methylotrophic yeasts are generally restricted to the use of methanol[13, 14]. Methanol is initially oxidized by alcohol oxidase (AOD) to formaldehyde

Formaldehyde is a central metabolic intermediate which is situated at the branching point between dissimilation and assimilation pathways in methanol metabolism[15].For the production of dihydroxyacetone and glyceraldehyde3-phosphate by dihydroxyacetone synthase (DAS), a portion of formaldehyde is directly fixed with xylulose5-phosphate. This enzyme is a transketolase-dependent type of thiamine pyrophosphate and uses xylulose5-phosphate and formaldehyde as physiological substrates. The products of the reaction are used to synthesize constituents of cells. Alternatively, to produce S-hydroxymethyl glutathione, formaldehyde reacts nonenzymatically with the reduced form of glutathione. The product is then successively oxidized to CO2 with NAD+- and glutathione-dependent formaldehyde

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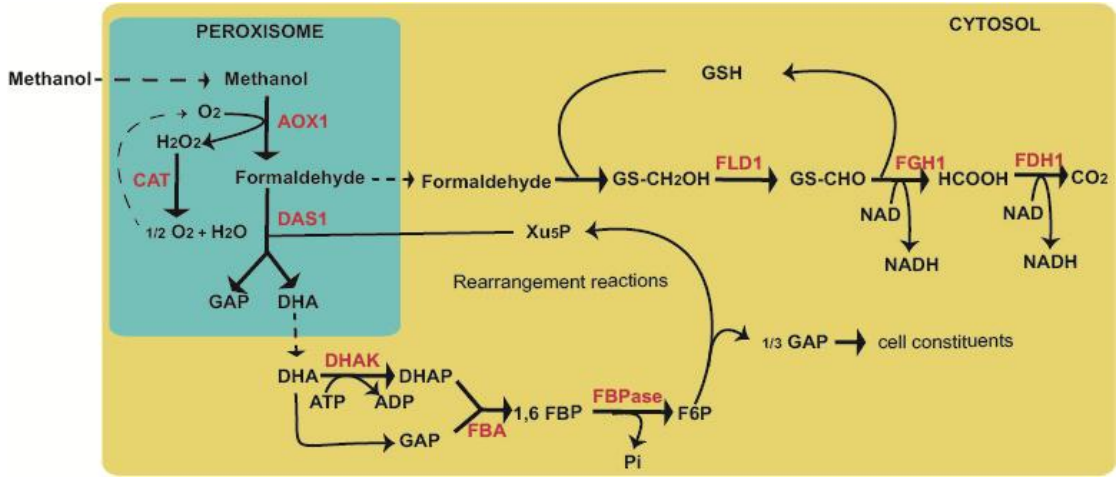
dehydrogenase (FLD), S-formylglutathione hydrolase (FGH), and NAD+-dependent format dehydrogenase (FDH). There are two physiological roles in this pathway: formaldehyde detoxification and generation of energy through NADH production[16-18]. The alcohol oxidase1 (AOX EC 1.1.3.13), which catalyzes the first step of the methanol utilization pathway (MUT pathway), can comprise up to 30% of the total soluble protein on methanol, showing the exceptional strength of the AOX1 promoter (pAOX1) (figure1)

On the contrary, the second alcohol oxidase AOX2 is controlled by a much weaker promoter (pAOX2) and thus accounts for only 15% of the overall AOX activity in the cell[19]. In P. pastoris, methanol metabolism is usually regulated by three mechanisms repression, derepression, and induction, where glucose is one of the substrates that act as a repressor of the methanol utilization pathway[20]. Glucose inhibits AOX1 promoter induction in P. pastoris recombinant. The induction power of PAOX1 is strongly dependent on methanol as the carbon source; this toxic and flammable material requires unique handling and is not appropriate for the production of edible and medical products. To understand more fully this valuable host organism, it is now necessary to understand the molecular mechanisms underpinning the unique carbon substrate utilization properties of P. pastoris[21]

Figure1: Outline of methanol metabolism in the methylotrophic yeast Pichia Pastoris.



AOD: alcohol oxidase; CAT: catalase; FLD1:formaldehyde dehydrogenase;FGH1: S-formylglutathione hydrolase FDH1:formate dehydrogenase, DAS: dihydroxyacetone synthase; DHAK:dihydroxyacetone kinase; FBA: fructose 1,6-biphosphatealdolase; FBPase fructose 1,6-bisphosphatase.

II. INTRODUCTION

The last thirty years of recombinant protein production in yeast has represented one of the great tools for synthetic biology and metabolic engineering applications. A methylotrophic yeast Pichia pastoris has proven to be the most commonly used host for the production of heterologous proteins for industrial interest[22]. *P. pastoris* is the best platform for this process because of a number of properties such as (i) the easy of techniques required for the molecular genetic manipulation of P. pastoris and their similarity to those of Saccharomyces cerevisiae, one of the most well-characterized experimental systems in modern biology; (ii) the capacity of P. pastoris to produce a variety of heterologous proteins at high levels, either extracellularly or intracellularly; (iii) as higher-eukaryotic the ability to carry out posttranslational modifications, such as glycosylation, disulfide bond formation and proteolytic processing; and (4i) the availability of the expression system as a commercially available kit[21, 23, 24]. This species of yeast is composed of two alcohols; Aox1 and Aox2; the two alcohols allow *Pichia* to use methanol as a source of carbon and energy. The methanol-inducible pAOX1, which is primarily attributed to the success of P. pastoris as a foreign protein expression system, was first isolated and used for the vector development and genetic manipulation protocols[20]. Controllable gene expression is an essential regulatory mechanism that can be achieved with depressed and inducible promoters. Most of these inducible promoters are susceptible to the repression of carbon catabolites or respond to other environmental factors, such as pressure, accumulation, or absence of essential amino acids, cell ion levels, and others[25-27]. Carbon catabolite repression is a crucial part of a global control system, adapts the carbon the metabolic

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machinery of the yeast P.pastoris to the utilization to a preferred carbon and energy source first through inhibition of synthesis of enzymes involved in the catabolism of carbon sources other than the preferred one[28, 29]. A comprehensive schema of the mechanism(s) of catabolite repression is not yet available, despite the accumulation of information on the subject compared to the model organisms E. coli and S. cerevisiae[30-32] where the solution of the puzzle has progressed, a little information is available on P. pastoris. Despite recent advances, the molecular regulation of methanol inducible promoters is still only partially understood.

III. LEVEL OF CONTROL

Controlled gene expression is often realized by inducible and repressed promoters[33]. Usually, many inducible promoters respond to catabolic repression. The catabolite repression in P.pastoris can be exerted by glucose but also by non-fermentable sources like glycerol, ethanol[34]. Glucose may affect enzyme levels by reducing the concentration of the corresponding mRNAs, reducing their rate of translation, or increasing the protein's rate of degradation. In turn, both the transcription rate of the corresponding gene and the stability of the mRNA would depend on the mRNA levels. The central regulation often occurs at the transcriptional level rather than the translational level[35]. In understanding the regulation of gene expression in Pichia pastoris, the responses of cells grown in glycerol, glucose, and methanol culture media are vital*[36, 37]*. The actual regulatory model usually involves three regulatory states, based on the observation of the promoter Paox1 induction, requiring the presence of the inducer methanol and the total lack of a repressing carbon source. The repression of catabolites suppresses expression as long as there is a repressive carbon source. The Paox1 is still being repressed even if methanol is being fed. Immediately the repressing carbon source is depleted, or at low concentration in fed-batch processes, the promoter reaches the state of derepression. In P. pastoris, under these conditions, the native AOX1 promoter is still only lightly derepressed (2–4% of induced levels), and the addition only of methanol alone leads to complete induction, which is the last and the third state of regulation[38, 39]. P. pastoris yeast's high cell densities are related to a shift from respiratory metabolism to respiro-fermentation[40] and reduced growth rates. The Crabtree-negative yeast P. pastoris retains its respiratory metabolism under conditions of excess glucose and shows comparable growth rates and substrates kinetics when cultivated on either glucose or glycerol[41]. When P. pastoris cells move from a growth medium containing glucose to a medium containing methanol as a single source of carbon, the yeasts undergo a drastic shift in the architecture of cells[42]. PAOX1's characteristics are vitally linked to the methanol expression regulator 1 (Mxr1) expression profile. Mxr1p has several functional similarities to *S. cerevisiae* Adr1p. Like Adr1p, Mxr1p is needed to activate multiple genes on more than one carbon source and is directly engaged in binding at least one of these target genes to the promoter.

IV. ELEMENTS OF SYSTEMS

The most comprehensive system of catabolite repression involves a parallel decrease in mRNA and protein levels[43]. Glucose has been reported to disturb the corresponding mRNAs in a few systems. Under the promoter paox1, most of the genes are controlled[44]. In order to control PAOX1, some downstream transcriptional activators (Mit1, Prm1, and Mxr1)[45, 46] and repressors like Nrg1(table), The Mig1 complex were recognized. In reaction to methanol, the transcription factors Mit1, Mxr1, and Prm1 positively controlled PAOX1 and bound to PAOX1 at separate locations and did not communicate with each other [47]

TABLE 1 **Promoter interacting elements of catabolite repression in P.pastoris(as reviewed in[48] [49] [50] [51] [52]**

Element	Designation	Function
Activator (DNA-binding proteins)	Mxr1	Activates transcription of genes for methanol metabolism
	Prm1	Positive transcription factor of P*AOX1*
	Mit1	Activates transcription of P*AOX1*

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Repressor (DNA-binding proteins)	Mig1 ,Mig2	Recruits Ssn6-Tup1 complex (repressor complex) in glucose repressed genes
	Nrg1	negative transcription factor of P*AOX1*
Glucose signalling	Hxt proteins	Hexose transporter
	Gss1	Impaired glucose catabolite repression

Mit1, methanol-induced transcription factor 1 in *P. pastoris* has a Zn(II)2Cys6-type similar to *H. polymorpha* Mpp1. The function analysis of different domains in mit1 shows low identity to H.polymorpha mpp1. Mit1 is a key PAOX1 regulator, acting downstream, and regulating PAOX1 tightly*[48]*. However, during methanol metabolism, he is not involved in peroxisome proliferation and transportation of peroxisomal proteins. Structural analysis by performing various domains deletions in Mit1 revealed its specific and essential function in the repression of P*AOX1* in glycerol medium. An in vivo assay demonstrates that the binding of Mit1 and Prm1 to P*AOX1* is carbon-dependent, and the binding strengths are low in glucose and glycerol but high in methanol. This suggests that glucose-related repressors or post-transcription alteration of Prm1 by phosphatase or kinases could repress the binding of Prm1 to PAOX1. This shows that for PAOX1 activation, Mit1 is essential than Prm1.

When the regulator factors Mit1, Prm1, or Mxr1 are removed, the transmission of the methanol induction signal to PAOX1 is inhibited. The methanol induction signal is transferred through a cascade between these regulator factors.Mxr1 is located in the cytoplasm of glucose-exposed cells but translocated to the nucleus of methanol, glycerol, and ethanol-exposed cells. This indicates that PAOX1's glucose-induced repression may be controlled by Mxr1's subcellular location variation. Other studies have also demonstrated that Mxr1 or its homolog, Trm2, derepresses methanol-inducible promoters[53]. There is no specific limit between induction and derepression, and Mxr1 may be involved in both of these processes.it has shown that Mxr1 is primarily engaged in PAOX1 derepression and is vital. Mxr1's role differed from that of Mit1 and Prm1, which replied mainly to methanol induction. MXR1 deletion blocked the methanol induction signal due to derepression failure. Accordingly, Aox expression could not improve by overexpression of Mit1 or Prm1.

The methanol signal was transferred to PMIT1 during the induction of methanol, leading in a drastic expression of Mit1. Further studies showed that Prm1 transferred the induction of methanol to PMIT1. Because cells exposed to glucose, glycerol, and methanol have demonstrated continuous Prm1 expression, Prm1 may have moved the methanol induction signal by changing its structure rather than changing the signal dose. Regulation of *P. pastoris* P*AOX1* by Mxr1, Prm1, and Mit1 is similar to the induction of oleate response genes meditated by Adr1, Oaf1, and Pip2. The transcription factors activated PAOX1 through a cascade. Mxr1 mainly acts during carbon derepression, while Prm1 and Mit1 operate during methanol induction, while Prm1 transmits methanol signal to Mit1 through binding to the MIT1 promoter (PMIT1), thus increasingly expressing Mit1 and activating PAOX1 afterward[54]. We also have repressors that play a crucial role in the repression by glucose. The Mig1 complex and Nrg1 are essential elements in glucose repression [49, 55].ScMig1, ScMig2, and ScMig3 are known in S.cerevisiae to repress the transcription of genes in the presence of glucose in other carbon source metabolism[56]. A P. pastoris genome BLAST search identified two homologs of ScMig: Mig1and Mig2 consist of 445 and 455 amino acids, and both belong to the Cys2His2 protein family near the N-terminus[57, 58]. The use of live-cell imaging of GFP tagged Mig1, or Mig2 indicated that Mig1 and Mig2 are predominantly located in the cytoplasm when P. pastoris cells are moved to methanol. This information suggested that Mig1 and Mig2 could be critical to PAOX1 catabolite repression. Besides, another significant PAOX transcription repressor Nrg1 was identified. The Nrg1 is a zinc finger protein involved in the glucose repression of several glucose-repressed genes. Since PpMxr1 is necessary for PAOX1 activation, PpNrg1 is likely competing with PpMxr1 binding locations to repress PAOX1 activation. In glucose and glycerol, Nrg1 suppressed PAOX1 by binding directly to five sites of PAOX1, including two binding sites of transcriptional activator Mxr1[50].

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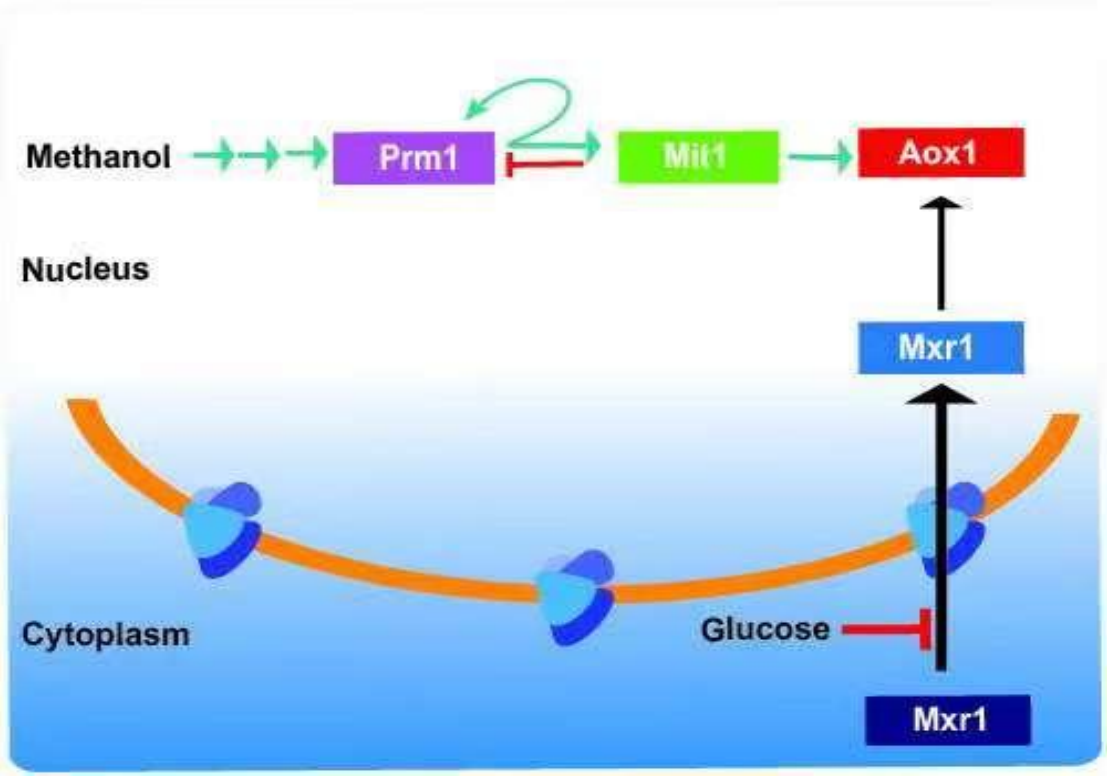

Figure 2. Regulatory model of PAOX1 activation by Mit1, Mxr1, and Prm1

PAOX1 is repressed in the presence of glucose because Mxr1 is located in the cytoplasm and has not shown the function of derepression. When using methanol as the unique source of carbon, Mxr1 is transported to the nucleus and relieved PAOX1's repression. The high expression of PAOX1 was induced by the methanol induction signal transmitted from Prm1 to Mit1 and then large amounts of Mit1. Prm1 induced the expression of itself during this progression, and Mit1 suppressed Prm1's expression.

V. CONCLUSION

The utilization of methanol as an inducer is confined to methylotrophic yeasts, which can metabolize methanol as a unique carbon source. This review describes the current state of catabolite repression in Pichia pastoris. Transcription activators Mxr1, Prm1, and Mit1, played essential roles in activating P_{AOX1} in response to methanol. Glucose repression is typically and widely exist in various organisms. Mit1, Prm1, and Mxr1 independently activate P AOX1 in P.pastoris. The Mig complex and Nrg1 repressors played an essential role in glucose repression.

The Mig complex and Nrg1 may regulate genes involved in the methanol pathways more finely and strictly when cells were grown in glucose than in glycerol. They may have redundant functions in transcriptional regulation. However, significant work still requires to be done to clarify the distinct processes of glucose-mediated repression.

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