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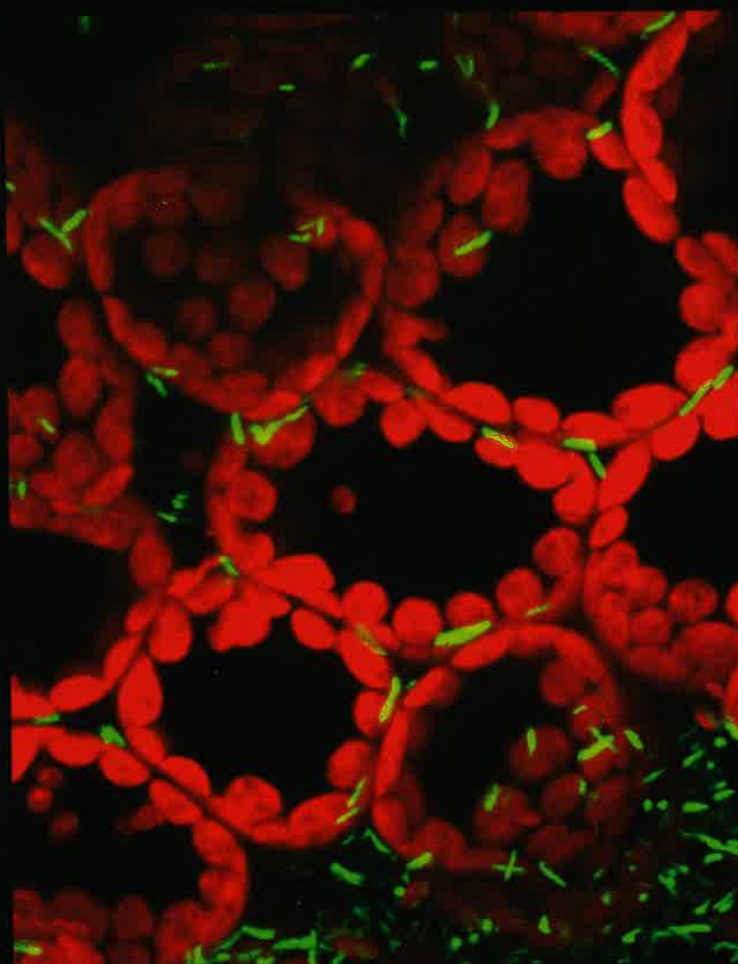
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Cover illustration: *Pseudomonas syringae* expressing GFP (green) can be seen in between tobacco plant cells. The chloroplasts (red) along the inner side of the plant plasma membrane outline the mesophyll cells.

For further details readers are referred to the article by Crabill *et al.* on pp. 225–238 of this issue.

***Pichia pastoris* 14-3-3 regulates transcriptional activity of the methanol inducible transcription factor Mxr1 by direct interaction**

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Summary

The zinc-finger transcription factor, Mxr1 activates methanol utilization and peroxisome biogenesis genes in the methylotrophic yeast, *Pichia pastoris*. Expression of Mxr1-dependent genes is regulated in response to various carbon sources by an unknown mechanism. We show here that this mechanism involves the highly conserved 14-3-3 proteins. 14-3-3 proteins participate in many biological processes in different eukaryotes. We have characterized a putative 14-3-3 binding region at Mxr1 residues 212–225 and mapped the major activation domain of Mxr1 to residues 246–280, and showed that phenylalanine residues in this region are critical for its function. Furthermore, we report that a unique and previously uncharacterized 14-3-3 family protein in *P. pastoris* complements *Saccharomyces cerevisiae* 14-3-3 functions and interacts with Mxr1 through its 14-3-3 binding region via phosphorylation of Ser215 in a carbon source-dependent manner. Indeed, our *in vivo* results suggest a carbon source-dependent regulation of expression of Mxr1-activated genes by 14-3-3 in *P. pastoris*. Interestingly, we observed 14-3-3-independent binding of Mxr1 to the promoters, suggesting a post-DNA binding function of 14-3-3 in regulating transcription. We provide the first molecular explanation of carbon source-mediated regulation of Mxr1 activity, whose mechanism involves a post-DNA binding role of 14-3-3.

Introduction

The methylotrophic yeast, *Pichia pastoris*, is employed as a model system for studying peroxisome biogenesis as

well as for the production of recombinant proteins (Cregg *et al.*, 1989; 2000; Cereghino and Cregg, 2000; Subramani *et al.*, 2000; Hartner and Glieder, 2006). Genes responsible for methanol catabolism (*AOX1*, *CAT* and *DHAS* encoding alcohol oxidase 1, catalase and dihydroxyacetone synthase, Aox, Cat and Dhas respectively) and peroxisome biogenesis (*PEX* genes) are induced by methanol and oleic acid (Cregg *et al.*, 1989; Hartner and Glieder, 2006). All three methanol utilizing enzymes are sequestered in peroxisomes where the initial reactions of methanol catabolism are compartmentalized, providing a rationale for the concurrent upregulation of methanol-utilizing enzymes and peroxisomes during growth in methanol (Gould *et al.*, 1992; Liu *et al.*, 1992; Johnson *et al.*, 1999; Stewart *et al.*, 2001). At the first step of methanol catabolism Aox oxidizes methanol to formaldehyde and the toxic by-product hydrogen peroxide is decomposed to oxygen and water by the action of Cat. *AOX1* expression is tightly regulated by carbon source being repressed by glucose, ethanol and glycerol and induced by methanol (Ellis *et al.*, 1985; Cregg *et al.*, 1989; Koutz *et al.*, 1989). Due to its efficient induction and tight regulation, the methanol-inducible promoter of *AOX1* (*P_{AOX1}*) has been employed in most heterologous recombinant protein over-expression systems in *P. pastoris* (Sakai *et al.*, 1995; Cereghino and Cregg, 2000; Cregg *et al.*, 2000; Gellissen, 2000).

Expression of the *MUT* (methanol utilizing) enzymes is regulated mainly at the transcriptional level by a zinc-finger transcription factor Mxr1 (methanol expression regulator 1) (Tschopp *et al.*, 1987; Cregg and Madden, 1988; Cregg *et al.*, 1989; Lin-Cereghino *et al.*, 2006). Functional disruption of Mxr1 causes a growth defect of *P. pastoris* in media containing methanol or oleic acid, and the expression of *AOX1* and *DHAS* was dramatically decreased. The oleic acid-induced expression of peroxisome biogenesis genes (i.e. *PEX5*, *PEX8*, *PEX14* and *FLD1*) was modestly affected and β -oxidation genes were affected hardly at all upon disruption of *MXR1* (Lin-Cereghino *et al.*, 2006). The binding sites for Mxr1 upstream of the *MUT* pathway and peroxisome biogenesis genes' promoters have been well characterized (Lin-Cereghino *et al.*, 2006; Hartner *et al.*, 2008; Kranthi *et al.*, 2009; 2010). A consensus sequence

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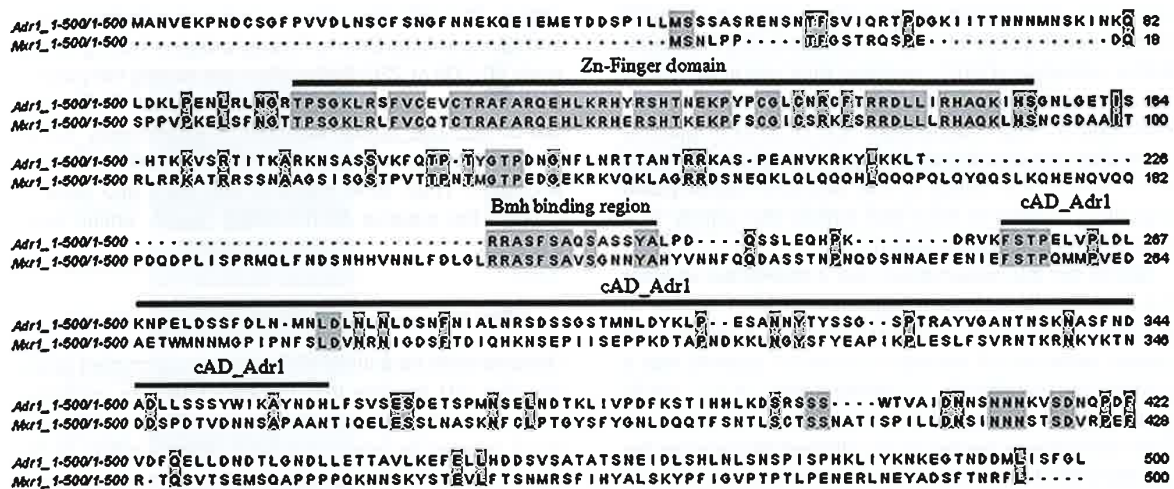


Fig. 1. Sequence alignment of Mxr1 and Adr1. The alignment was done using web server based alignment program, ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) (Larkin *et al.*, 2007) by taking 1–500 amino acids of both the proteins. cAD, cryptic activation domain; Zn-Finger, DNA binding domain.

consisting of a core 5'-CYCC-3' motif is recognized by Mxr1 (Kranthi *et al.*, 2009). Despite the constitutive expression of Mxr1 in glucose grown cells its activity remains repressed, suggesting the existence of post-translation modification(s) that regulate its activity (Lin-Cereghino *et al.*, 2006). However, any post-translation modification(s) that might regulate Mxr1 activity in response to glucose is unknown. Based on immunofluorescence studies of a Mxr1-GFP fusion protein it was proposed that decreased nuclear localization of Mxr1 in glucose grown cells might be an explanation for glucose repression of Mxr1-dependent AOX1 and other methanol-inducible genes (Lin-Cereghino *et al.*, 2006). However, it is unknown why Mxr1 remains inactive in ethanol- and glycerol-grown cells despite its nuclear localization in those growth conditions (Lin-Cereghino *et al.*, 2006).

As observed by Lin-cereghino *et al.* (2006), the N-terminally located C₂H₂ zinc-finger DNA binding domain (residues 29–101) of Mxr1 shares ~ 70% sequence identity with the DNA binding domain (residues 95–156) of *Saccharomyces cerevisiae* Adr1 (alcohol dehydrogenase II synthesis regulator), a transcription factor regulating the expression of glucose-repressed genes involved in the metabolism of non-fermentable carbon sources such as glycerol, lactate, amino acids, ethanol and oleic acid (Ciriacy, 1979; Denis *et al.*, 1981; Hartshorne *et al.*, 1986; Simon *et al.*, 1992; 1995; Young *et al.*, 2003). Despite the high sequence similarities with the DNA binding region of Adr1, Mxr1 does not show any cross-reactivity for Adr1-binding UAS (upstream activator sequence) elements (Kranthi *et al.*, 2009). Outside of their DNA binding regions, which share a similar location, Mxr1 and Adr1 share little

homology, despite their similar size and analogous function in regulating the metabolism of non-fermentable substrates. The only other region of homology within Mxr1 is a short stretch of amino acids which shares approximately 70% sequence identity (Fig. 1) with the core 14-3-3 (Bmh) binding region (Conserved region 1) (Parua *et al.*, 2010) located within the regulatory domain of Adr1.

14-3-3 proteins are ubiquitous, highly conserved, acidic and dimeric proteins that play important roles in controlling a wide variety of cellular processes, including cell cycle, metabolism, apoptosis and gene expression by binding to phosphoserine- or phosphothreonine-containing motifs in numerous target proteins (Jones *et al.*, 1995; Xiao *et al.*, 1995; Muslin *et al.*, 1996; Yaffe *et al.*, 1997; Fu *et al.*, 2000; Ferl *et al.*, 2002; Mackintosh, 2004; Wilker *et al.*, 2005; Aitken, 2006; van Heusden and Steensma, 2006). The yeast *S. cerevisiae* has two genes encoding redundant 14-3-3 proteins, Bmh1 and Bmh2 (Gelperin *et al.*, 1995; van Heusden *et al.*, 1995; van Heusden and Steensma, 2006). Either Bmh protein can fulfil all of the essential functions in budding yeast. As in higher eukaryotes, yeast 14-3-3 proteins are involved in numerous signalling and cell differentiation pathways, including glucose repression. 14-3-3 proteins have not been reported in *P. pastoris* but their ubiquitous presence in other eukaryotes makes it likely that this yeast also contains at least one homologue which may have functional similarities to Bmh in *S. cerevisiae*.

Bmh in *S. cerevisiae* is involved in glucose repression at two levels. By binding to Reg1, the regulatory subunit of the Glc7 protein phosphatase, Bmh participates in the inactivation of the AMP-activated protein kinase (AMPK) Snf1

when cells are grown in glucose (Mayordomo *et al.*, 2003; Dombek *et al.*, 2004; Ichimura *et al.*, 2004). Snf1 is essential for activation of Adr1, and thus Bmh indirectly inhibits the expression of Adr1 target genes. Recently we described a Reg1-independent role of Bmh in glucose repression that acts directly through binding to Adr1 (Parua *et al.*, 2010). Bmh binds to the Ser230-phosphorylated regulatory domain of Adr1 and inhibits the activity of a nearby cryptic activating region.

Based on this observation, we investigated whether there is 14-3-3-dependent regulation of Mxr1 activity in *P. pastoris*. We show that Mxr1 contains a highly conserved yeast 14-3-3 binding motif and *P. pastoris* has a unique and previously uncharacterized 14-3-3 family protein that interacts with Mxr1 through its 14-3-3 motif. The interaction is necessary to differentially regulate the activity of Mxr1 depending on the available carbon source. We provide evidence that 14-3-3-mediated regulation of transcription occurs at a step after DNA-binding. We also demonstrate that the first 400 amino acids of Mxr1 are sufficient for carbon source-mediated repression and activation of Mxr1-regulated genes and map the major activation domain of Mxr1 to this region of the protein. The *P. pastoris* 14-3-3 is able to complement a budding yeast strain with defective Bmh genes, indicating that it can fulfil the essential functions of Bmh in *S. cerevisiae*.

Results

S. cerevisiae 14-3-3 interacts with Mxr1

Besides the closely related C₂H₂ Zn-finger DNA binding domains (Lin-Cereghino *et al.*, 2006), the sequence alignment (Fig. 1) between residues 1–500 of both Mxr1 and Adr1 reveals the presence of only one other region of homology (~70% sequence identity) corresponding to the Bmh1/2 (*S. cerevisiae* 14-3-3 homologues) binding region of Adr1, residues 226–240. This putative 14-3-3 binding site (-RRASFS-----YA-) is located between amino acids 212–225 of Mxr1. Based on this sequence analysis we asked whether *S. cerevisiae* 14-3-3 proteins can interact with Mxr1 through the observed putative binding motif. To answer this question, five GBD (Gal4 DNA binding domain)-Mxr1 fusion proteins, encompassing different regions of Mxr1 were generated and tested in pulldown assays with Gst (Glutathione-S-transferase)-Bmh1. As shown in Fig. 2A, GBD-Mxr1 proteins (encompassing amino acids 96–266, 206–246 and 206–226 of Mxr1) having the putative Bmh binding region were retained on glutathione sepharose 4B-immobilized Gst-Bmh1 resin. The fusion protein GBD-Mxr1 (206–226) showed low binding to the Gst-Bmh1 beads compared with the other two. A possible explanation is that the short 20-residue-long peptide might not be fully accessible in the fusion

protein. This indicates a physical interaction between Mxr1 and *S. cerevisiae* Bmh1. Fusion proteins having amino acids 96–206 or 226–266 of Mxr1 but lacking the putative Bmh binding region, did not exhibit any interaction with Gst-Bmh1. The empty Gst-control experiments further confirmed the specificity of the interaction between Bmh1 and Mxr1. Thus, Bmh interacts with Mxr1 and does so through the putative Bmh binding region, amino acids 212–225.

14-3-3 of *P. pastoris* binds to Mxr1

Genome-wide searching and sequence alignment analyses (Fig. S1) indicate that *P. pastoris* has an uncharacterized 14-3-3 family protein, known as C4qzn3 (257 residues) located on chromosome 2. This putative 14-3-3 shares almost 90% sequence identity with 14-3-3 proteins from other eukaryotes, *S. cerevisiae*, *S. pombe*, *C. albicans*, *D. melanogaster*, plants and human (Fig. S1). Gst-pulldown experiments were performed using Gst-C4qzn3 or Gst-Bmh1 expressed in *E. coli* and yeast extracts containing GBD-Mxr1 or GBD-Adr1 fusion variants to assess their possible interaction. Comparable amounts of both GBD-Adr1 (215–260) and GBD-Mxr1 (210–226) were retained on Gst-C4qzn3-bound resin as observed for GBD-Mxr1 (210–226) on Gst-Bmh1-bound resin (Fig. 2B). GBD-Mxr1 (96–206), lacking the Bmh binding region was not pulled down with Gst-C4qzn3, suggesting that the interaction was specific for the presence of the Bmh binding region (residues 212–225) in Mxr1. These results demonstrate that the putative *Pichia pastoris* 14-3-3 protein has the ability to interact with both transcription factors, *P. pastoris* Mxr1 and *S. cerevisiae* Adr1 at their respective Bmh binding regions.

14-3-3 interacts with Mxr1 via phosphorylation of Ser215

14-3-3 proteins preferentially recognize Ser/Thr-phosphorylated targets and in our previous work we observed that Bmh interacts with Adr1 via phosphorylation of Ser230, which is located within the core Bmh binding region (Parua *et al.*, 2010). The sequence alignment in Fig. 1 shows that Ser215 of Mxr1 is the residue corresponding to Ser230 of Adr1, which is known to be phosphorylated in a carbon-source regulated manner. To determine if the phosphorylation status of Ser215 plays a role in 14-3-3 binding to Mxr1, we first examined the phosphorylation status of Ser215 by performing immunoblotting experiments with anti-pSer230 antibody raised against the homologous phosphorylated Bmh1 binding motif of Adr1 (Ratnakumar *et al.*, 2009). As shown in Fig. 2B, the anti-pSer230 antibody reacted with the pulldown fraction of GBD-Mxr1 (210–226) and GBD-Adr1

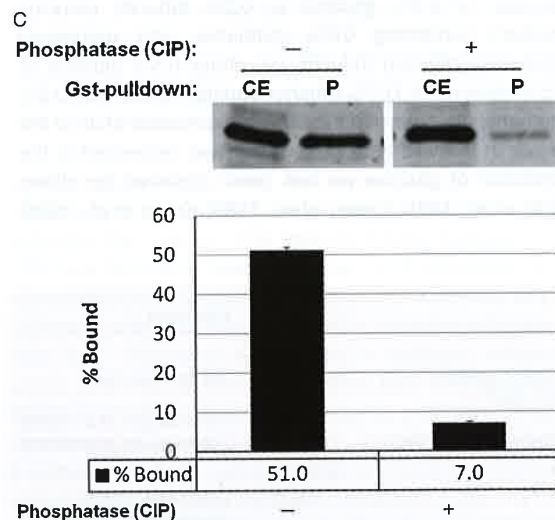
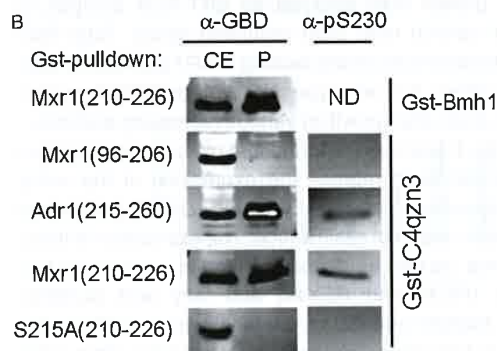
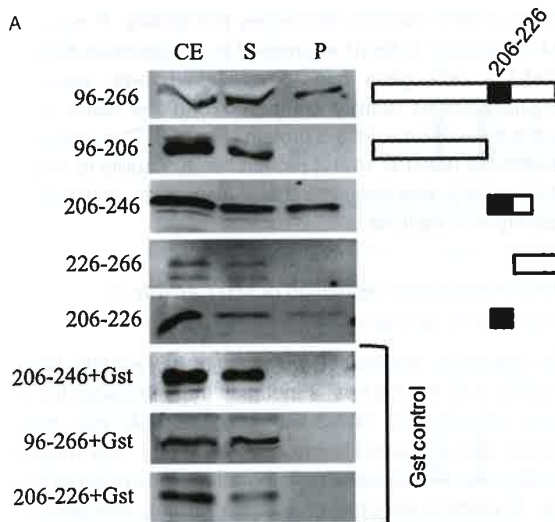


Fig. 2. A. Interaction studies between Mxr1 and Bmh1. Gst-pulldown assays were done using Gst-Bmh1 or Gst, which was expressed in *E. coli* and immobilized on glutathione sepharose-4B column, followed by application of yeast extract containing overexpressed GBD (Gal4 DNA binding domain)-Mxr1 fusion protein. All fractions were electrophoresed in 12% SDS-PAGE and visualized by Western blotting with anti-GBD antibody. Black shaded region (residues 206–226) represents the putative Bmh binding region. B. Interaction studies of C4qzn3 with *Pichia pastoris* Mxr1 and *S. cerevisiae* Adr1. Gst-pulldown assays were done using Gst-Bmh1, Gst-C4qzn3. Yeast extracts containing either overexpressed GBD-Mxr1 or GBD-Adr1 variants was used in this study. All fractions were electrophoresed in 15% SDS-PAGE and visualized by Western blotting with anti-GBD antibody or anti-pSer230 antibody. C. Gst-pulldown in presence and absence of phosphatase. Gst-pulldown was done following the protocol as described before using Gst-C4qzn3 and yeast extract containing GBD-Mxr1 (210–226) fusion protein in presence and absence of 10U of calf intestinal phosphatase (CIP) at 30°C. Upper panel is the Western blot profile of the pulldown fractions obtained by using anti-GBD antibody and lower panel represents the quantitative assays of the Western blot results. CE, Cell extract; S, Supernatant; P, pellet fraction.

(215–260), and not with cell extract of GBD-Mxr1 (96–206), suggesting that Ser215 within the 14-3-3 motif is phosphorylated in *S. cerevisiae*. To determine the role of pSer215 in binding of 14-3-3 to Mxr1, Ser215 was substituted with Ala in GBD-Mxr1 (210–226) and Gst-pulldown assays were performed. As shown in Fig. 2B the S215A mutant was neither pulled down nor recognized by the anti-pSer230 antibody suggesting that phosphorylation of Ser215 is essential for 14-3-3 binding. The phosphorylation-dependent interaction between 14-3-3 and Mxr1 was further confirmed by performing Gst-pulldown assays using Gst-immobilized *P. pastoris* 14-3-3 and yeast extract containing fusion protein GBD-Mxr1 (210–226) following the same protocol as described before in presence and absence of 10U of calf intestinal phosphatase (CIP; NEB) at 30°C. As shown in Fig. 2C, the phosphatase treatment significantly reduced the interaction, suggesting that the phosphorylation of Mxr1 is important for binding by 14-3-3 and most likely pSer215 is the target.

14-3-3 protein of *P. pastoris* complements Bmh function(s) in *S. cerevisiae*

The ability of the *P. pastoris* 14-3-3 protein to complement the loss of 14-3-3 function in *S. cerevisiae* would demonstrate that C4QZN3 encodes a functional homologue. First, the ability of N-terminally HA-tagged *P. pastoris* 14-3-3 (from pBF-C4QZN3) or Bmh1 (from pBF-BMH1) to complement the growth defect of a *bmh2Δ bmh1-ts* mutant strain at 37°C was examined. As shown in Fig. 3A, expression of *P. pastoris* 14-3-3 rescued growth at 37°C as well as Bmh1, suggesting that *P. pastoris* 14-3-3 can perform all of

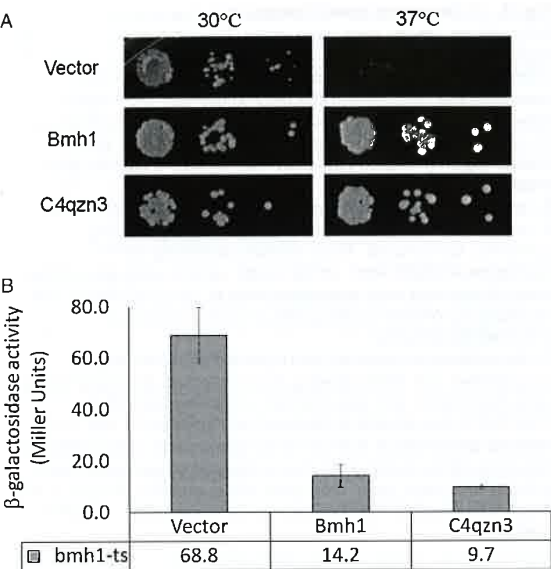


Fig. 3. A. Growth test of *bmh2Δ bmh1-ts* mutant strain expressing either HA-tagged *Pichia* 14-3-3 (C4qzn3) or Bmh1 at 30°C and 37°C on Trp⁻ plates. B. Evaluation of the activity of endogenous Adr1 by exploiting a reporter gene (*ADH2p-lacZ*) expression assays. *S. cerevisiae bmh2Δbmh1-ts* cells harbouring a pair of plasmids, one expressing either *P. pastoris* 14-3-3 or Bmh1 or none (empty vector), another providing the reporter construct (*pLG-ADH2-lacZ*; *lacZ* under the control of *ADH2* promoter) were used as host and grown at 30°C. The error bars represent the averages of the results for three transformants ± one standard deviation.

the essential functions of the *S. cerevisiae* 14-3-3 proteins. Next, the regulatory effect of *P. pastoris* 14-3-3 protein on the transcriptional activity of Adr1 was tested. We evaluated the activity of endogenous Adr1 by assaying expression of the Adr1-dependent reporter gene, *ADH2p-lacZ* in *bmh2Δ bmh1-ts* (YLL1087) strain. The expression of the reporter is inhibited by Bmh due to its binding to the Adr1 regulatory region and consequent inhibition of Adr1 activity. Loss of Bmh activity results in a high level of reporter expression and thus high β-galactosidase activity. Resto-

ration of Bmh function decreases this activity. *P. pastoris* 14-3-3 protein or Bmh1 expressed in *S. cerevisiae bmh2Δ bmh1-ts* cells gave rise to a fivefold lower level of β-galactosidase activity compared with the same cells without a wild-type 14-3-3 protein (Fig. 3B). This indicates that the *P. pastoris* 14-3-3 protein has the ability to inhibit Adr1 activity and suggests that it also might inhibit Mxr1 activity in *P. pastoris*.

14-3-3-dependent regulation of Mxr1 activity in response to various carbon sources

To determine whether *P. pastoris* 14-3-3 inhibits Mxr1 activity in its natural host a mutation that abrogates the *in vitro* interaction of 14-3-3 with Mxr1 (S215A) was introduced into a plasmid-borne copy of *MXR1*. The mutant protein was expressed after integration of the plasmid into the *P. pastoris* genome of a strain lacking endogenous *MXR1* (JC132, Table 1). The activity of wild-type and mutant protein was assayed by qRT-PCR analysis of mRNAs derived from Mxr1-regulated genes. Total RNA was extracted from strains lacking *MXR1* (JC132), or containing either the wild-type (JC100) or S215A mutant (PPP1) allele after growth in various repressing and inducing media. It has been reported that glucose, glycerol, and ethanol can each repress the expression of the solely Mxr1-dependent genes *AOX1* and *DHAS*, which are involved in methanol assimilation. The expression of these two genes, as well as genes involved in peroxisome biogenesis (*PEX8* and *PEX14*) and fatty acid oxidation (*POT1*, thiolase and *POX1*, oxidase) was quantified after growth in fully repressing medium containing either 0.5% glucose, or 0.5% glycerol or 0.5% ethanol; inducing medium containing 0.5% methanol; and repressing medium containing inducer, i.e. either 0.5% glucose or 0.5% glycerol or 0.5% ethanol supplemented with 0.5% methanol. As shown in Fig. 4A–C, expression of all of the genes in the wild-type Mxr1 strain was repressed in the presence of glucose as has been observed by others (Ellis *et al.*, 1985; Cregg *et al.*, 1989; Koutz *et al.*, 1989;

Table 1. Yeast strains.

Strain	Genotype	Reference
<i>S. cerevisiae</i>		
PJ69-4a	<i>MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4Δ gal80Δ LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ</i>	James <i>et al.</i> (1996)
YLL908	W303 <i>MATa bmh2Δ::kanmx</i>	Lottersberger <i>et al.</i> (2003)
YLL1087	W303 <i>MATa bmh2Δ::kanmx bmh1Δ::HIS3::Ylpbmh1-170(LEU2)</i>	Lottersberger <i>et al.</i> (2003)
<i>P. pastoris</i>		
JC100	Wild type	Cregg <i>et al.</i> (1998)
JC132	<i>mxr1-1 his4</i>	Johnson <i>et al.</i> (1999)
PPP1	<i>MXR1-S215A</i>	This study
PPP2	<i>MXR1tm-S215A</i>	This study
PPP3	<i>MXR1tm</i>	This study

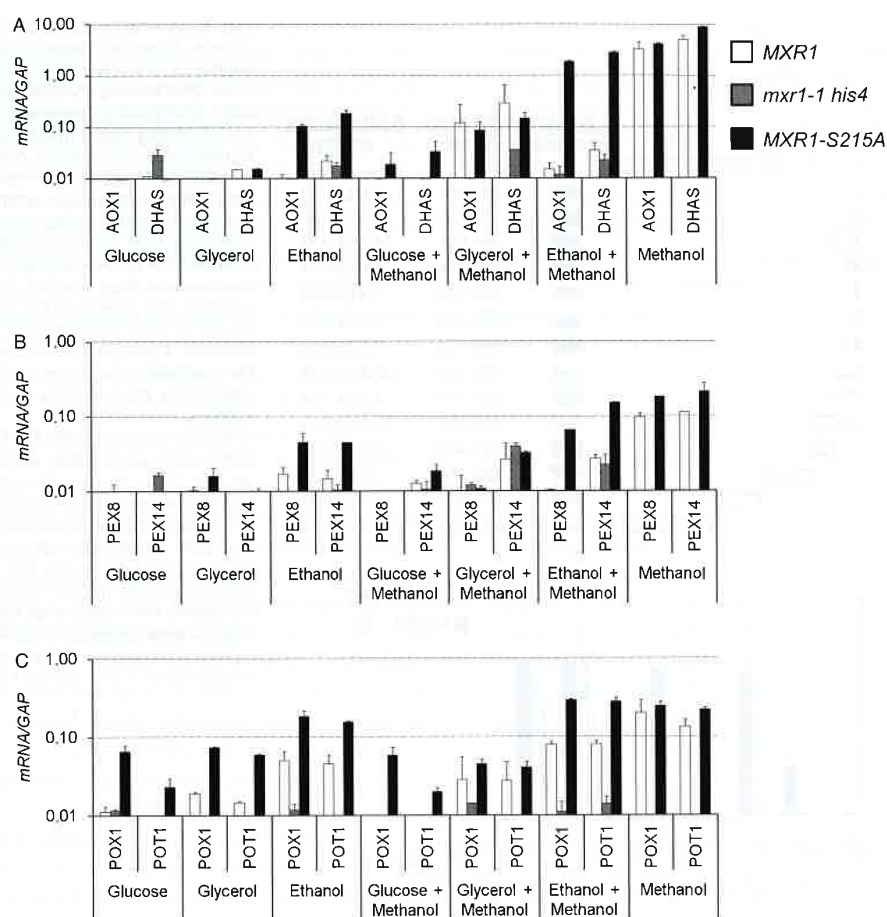


Fig. 4. Studies on Mxr1-dependent expression of genes involved in methanol utilization (*AOX1* and *DHAS*), peroxisome biogenesis (*PEX8* and *PEX14*) and fatty acid oxidation (*POT1* and *POX1*) in *P. pastoris* in response to different carbon sources. mRNA was extracted from strains JC100 (*MXR1*WT), JC132 (*mxr1-1 his4*), PPP1 (*MXR1-S215A*) both in repressed (0.5% glucose, 0.5% glycerol and 0.5% ethanol) and induced (0.5% methanol) as well as repressed plus methanol growth conditions. Quantitative RT-PCR (qRT-PCR) was performed to determine the levels of mRNA. The mRNA levels were normalized to the levels of *GAP* mRNA in each sample. The error bars represent the mean of three biological replicates assayed in duplicate.

Lin-Cereghino *et al.*, 2006). Moreover, glycerol-mediated repression of all of these genes was also observed in the wild-type Mxr1 strain. This was surprising because the *PEX* and fatty acid oxidation genes of *S. cerevisiae* are derepressed when glycerol is the sole carbon source. *MUT* pathway genes and genes involved in peroxisome biogenesis were repressed significantly in wild-type ethanol grown cells (Fig. 4A and B). However, fatty acid oxidation genes were not repressed in ethanol (Fig. 4C), which is analogous to Adr1-dependent derepression of the β -oxidation pathway genes in ethanol media in *S. cerevisiae*. Furthermore, their complete repression in the *mxr1-1* null mutant strain suggests that expression of β -oxidation pathway genes is highly Mxr1-dependent.

Interestingly, there was derepression of the expression of fatty acid oxidation genes (~ 7- to 8-fold in glucose media

and ~ 2- to 3-fold in glycerol media), methanol assimilation pathway genes (more than 10-fold in ethanol growth condition) and peroxisome biogenesis genes (~ 2- to 3-fold in ethanol media) in the strain carrying the S215A mutant allele, suggesting that disruption of the interaction with 14-3-3 reduces carbon source-inhibited transcriptional activation by Mxr1 (Fig. 4). Addition of methanol into the ethanol growth media further increased the expression of the *AOX1*, *DHAS* and *PEX14* in the S215A mutant strain to a level comparable to that observed in inducing condition (Fig. 4A and B). This suggests the existence of at least two layers of control over the expression of *MUT* and *PEX* genes. All of the genes tested remained repressed after addition of methanol to the glucose media (Fig. 4A–C). However modest induction was observed for *MUT* pathway and β -oxidation genes in the wild-type strain upon

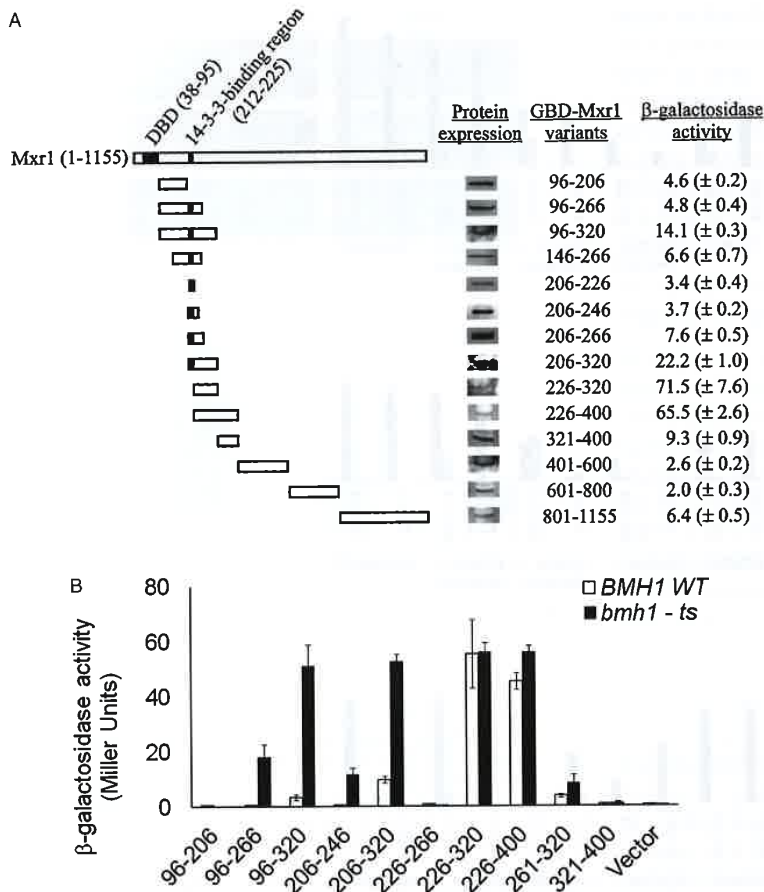


Fig. 5. A. Activity assays of GBD-Mxr1 variants. Diagram represents the various Mxr1 fragments, which were generated as GBD (Gal4 DNA binding domain) fusion protein. DBD, DNA binding region and the conserved 14-3-3 binding region (residues 212–225) are highlighted by black shade. Activity was measured by exploiting the reporter gene (*lacZ*) expression assays, where *S. cerevisiae* PJ69-4a strain harbouring plasmid, which expressed Mxr1 variants as GBD fusion proteins. The strain was also harbouring a chromosomal copy of a *GAL7p-lacZ* reporter cassette. The values are the averages of the results for three transformants ± one standard deviation. Expression level of all GBD-fused Mxr1 peptides was also shown in the figure obtained by Western blotting using anti-GBD antibody.

B. Activity assays of GBD-Mxr1 proteins in *BMH1* wild type (YLL908; *bmh2Δ BMH1*) and *bmh1-ts* (YLL1087; *bmh2Δ bmh1-170*) strain. Activity was assayed by measuring the activity of expressed β-galactosidase from *lacZ* gene, where cells harbouring a pair of plasmids, one having *GAL10p-CYC1-lacZ* reporter cassette (pHZ18') and another expressing Mxr1 variants as GBD fusion proteins were grown at 30°C. The error bars represent the averages of the results for three transformants ± one standard deviation.

addition of methanol to glycerol-containing medium (Fig. 4A and C), suggesting that glucose and glycerol repress Mxr1 activity through distinct pathways. In the *mxr1-1* null mutant none of the genes were expressed in any growth condition and all of them were significantly induced in *MXR1* wild-type and *S215A* mutant cells when methanol was the sole carbon source. In conclusion, a mutation that disrupts the interaction of 14-3-3 with Mxr1 allows significant methanol-independent expression of Mxr1-dependent genes.

Mapping of activation domain(s) of Mxr1

Although Mxr1 is an important transcription factor for the expression of genes involved in methanol utilization, oleic acid metabolism and peroxisome biogenesis, the existence and location of activation region(s) of the protein have not been identified. Sequence comparison to Adr1 did not identify a region with significant homology to any of its activation domains. To identify and map potential activation domains of Mxr1 we employed yeast one-hybrid assays

where GBD-fused variants of Mxr1 (Fig. 5A) were expressed in *S. cerevisiae* strain PJ69-4a (Table 1) and their activity was assayed by evaluating the expression of three Gal4-dependent reporter genes, *GAL2p-ADE2*, *GAL1p-HIS3* and *GAL7p-lacZ*. As shown in Fig. 5A, the highest β-galactosidase activity was observed for the GBD-Mxr1 fusion proteins encompassing residues 226–320 and 226–400, suggesting that a major activation domain is located between residues 226–320. Despite the presence of residues 226–320 the fusion proteins with amino acids 96–320 and 206–320 showed approximately 3–5 times lower activity. Interestingly, both of the Mxr1 fusions contain the 14-3-3 binding region (residues 212–225), while fragments, 226–320 and 226–400 do not, suggesting that the 14-3-3 binding region acts to inhibit the function of this activation domain. Furthermore, as shown in Fig. 5A, all of the GBD-Mxr1 fusion proteins were expressed significantly, suggesting that the observed activity was not a reflection of the level of expression and the stability of a peptide. The results of the β-galactosidase assays were confirmed by measuring the activity of the

GAL1p-HIS3 and *GAL2p-ADE2* reporter genes by observing the growth on Trp⁻, Trp⁻Ade⁻ and Trp⁻His⁻ plates (Fig. S2). Only cells expressing GBD-Mxr1 variants encompassing regions 226–320 or 226–400 showed substantial growth on both Trp⁻Ade⁻ and Trp⁻His⁻ plates, confirming the location of a strong activation domain at amino acids 226–320. Consistent with the observed low level of β -galactosidase activity, the fusion proteins encompassing residues, 96–320 and 96–400 showed reduced growth on Trp⁻His⁻ and no growth on Trp⁻Ade⁻ plates. As both of the above two fusion proteins contain the observed Bmh binding region (212–225 amino acids), supporting an inhibitory role of the 14-3-3 binding region on activation domain function. Moreover our results indicate the presence of a strong activation region(s) located in the vicinity of the N-terminus just downstream of the zinc-finger DNA binding domain.

Our hypothesis that the 14-3-3 binding region inhibits the major activation domain of Mxr1 was confirmed by evaluating the activity of selected GBD-fused Mxr1 variants in both *BMH1* wild type (YLL908; *bmh2 Δ BMH1*) and *bmh1-ts* mutant (YLL1087; *bmh2 Δ bmh1-170*) strains using the *GAL10p-CYC1-lacZ* reporter gene at 30°C, a temperature sufficiently high to cause a *bmh⁻* phenotype (Parua *et al.*, 2010). In both *bmh2 Δ BMH1* and *bmh2 Δ bmh1-ts* mutant strains we observed comparable amounts of activity for the GBD-Mxr1 (226–320) and GBD-Mxr1 (226–400) fusion proteins, which lack the Bmh binding region (Fig. 5B). Fusion proteins containing both the Bmh binding region and all or part of the activation region (96–266, 96–320, 206–246 and 206–320), were approximately 5- to 15-fold more active in the *bmh2 Δ bmh1-ts* mutant than in the wild-type strain (Fig. 5B). All other fusion proteins tested had little or no activity in both strains. The absence of activity for the fusion protein encompassing residues 226–266 and the low activity of the 261–320 fragment suggests the critical role of the entire 226–320 region for the activity of Mxr1. Inactivation of Bmh or removal of the 14-3-3 binding region suppressed the inhibition of activation. This suggests that the binding of 14-3-3 to amino acids 212–225 of Mxr1 inhibits the activity of the major activation domain located at residues 226–320.

Characterization of the major activation domain of Mxr1

To map the activation domain more precisely we generated additional GBD-Mxr1 fusion proteins spanning residues 226–320 of Mxr1 and measured their activity in strain PJ69-4a. The results of the β -galactosidase (Fig. 6A) and growth assays (Fig. 6B) indicate that the shortest region capable of conferring activating function is located between amino acids 246 and 280.

Interestingly, the shortest functional region of Mxr1 (residues 246–280) is enriched in hydrophobic and acidic resi-

dues (see Fig. 1), characteristics of canonical activation domains in which bulky hydrophobic residues are frequently important for activation domain function (Cress and Triezenberg, 1991; Regier *et al.*, 1993; Blair *et al.*, 1994; Gill *et al.*, 1994; Lin *et al.*, 1994; Drysdale *et al.*, 1995; Folkers *et al.*, 1995; Almlöf *et al.*, 1997; Young *et al.*, 1998). The importance of the three phenylalanine residues located within this region of Mxr1 at positions 249, 254 and 278 was tested by substituting them by Ala either individually or in combination. All of the mutants were expressed as GBD-fusion proteins in *S. cerevisiae* strain PJ69-4a and their activity was measured by means of β -galactosidase activity and growth assays as described above. A threefold decrease in *GAL7p-lacZ* expression was observed when individual Phe residues were changed to Ala. Substitution of all three to Ala caused an approximately fivefold decrease in expression compared with the wild-type fusion protein (Fig. 6C). Furthermore, the cells expressing either a single, double, or triple mutant protein were inviable on Trp⁻Ade⁻ medium (Fig. 6D). Western blot analysis of the GBD fusion proteins indicated that all the mutant proteins were expressed at a significant level (Fig. 6C). These observations corroborate the importance of the hydrophobic Phe residues for the activity of the GBD-Mxr1 fusion protein and show that this region of Mxr1 functions as a canonical activation domain.

The N-terminal 400 amino acids of Mxr1 are sufficient for full transcriptional activity in *P. pastoris*

We have shown that the functions of Mxr1 required for activating transcription in *S. cerevisiae* are located within the N-terminal 400 amino acids of the protein. Because this region of Mxr1 also contains its DNA binding domain we could determine whether it is sufficient for activation of Mxr1 target genes in its natural host, *P. pastoris*. A plasmid carrying the *MXR1* promoter followed by either the *S215A* mutant or wild-type coding sequence truncated to amino acid 400 was introduced into the *his4* gene of *P. pastoris* JC132 to create strain PPP2 (*MXR1tm-S215A*) and PPP3 (*MXR1tm*) respectively. These strains as well as a strain expressing the full-length wild-type protein, JC100, a *mxr1* null mutant strain, JC132, and a strain expressing the full-length Mxr1-S215A mutant protein, PPP1 were grown in repressing medium, 0.5% ethanol, inducing medium, 0.5% methanol, and repressing medium containing inducer, 0.5% ethanol and 0.5% methanol. Total RNA was isolated from each culture and *AOX1* mRNA, as a measure of Mxr1 activity, was quantified using qRT-PCR. As shown in Fig. 7, the truncated Mxr1 wild type (Mxr1tm-WT) and mutant (Mxr1tm-S215A) displayed a level of *AOX1* expression comparable to that found in the full-length wild type and Mxr1-S215A mutant in inducing condition. Furthermore, both S215A mutants exhibited comparable levels of

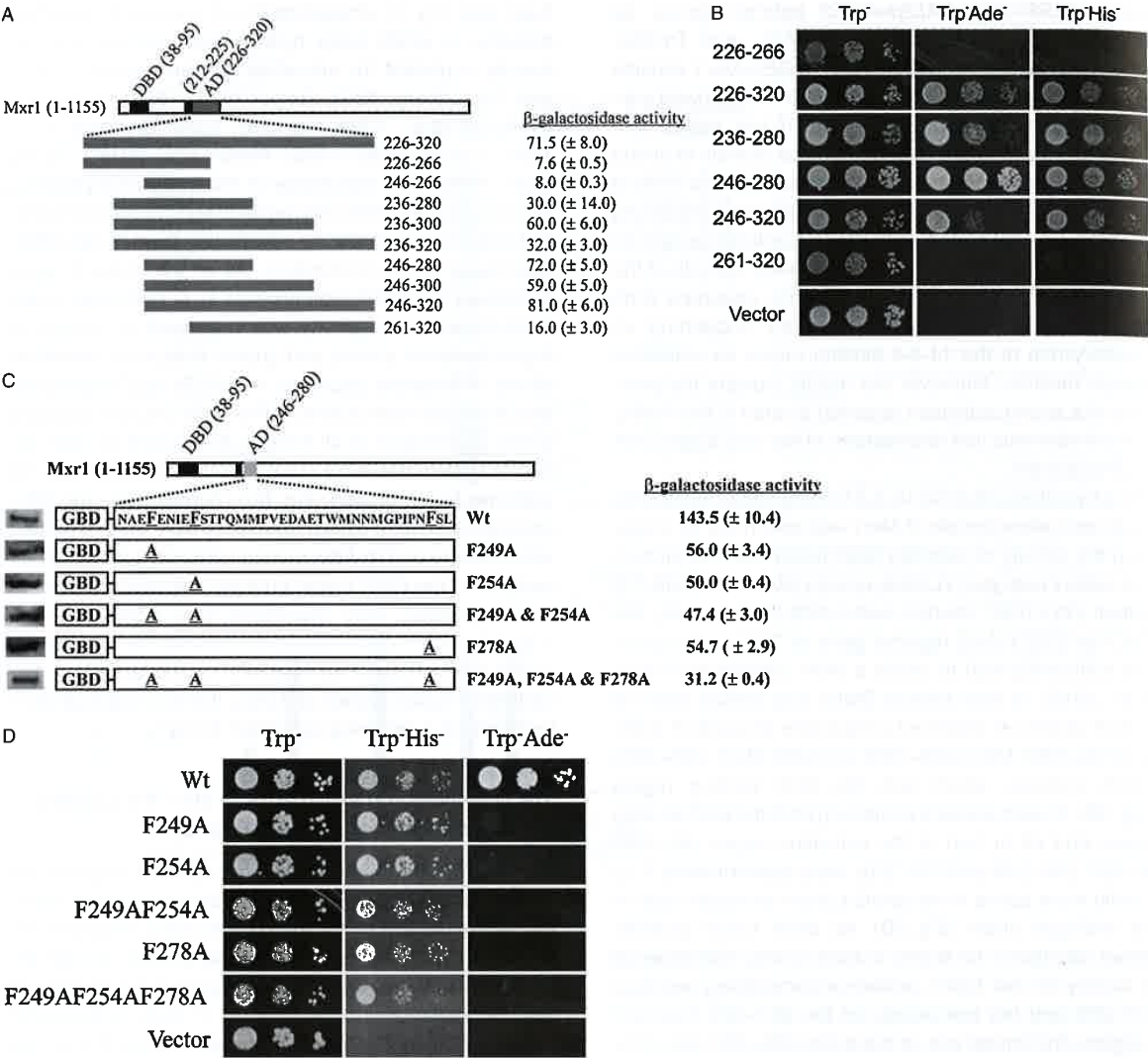


Fig. 8. A. Indicated different Mxr1 fragments encompassing the 226–320 amino acids region, were expressed as GBD (Gal4 DNA binding domain) fusion protein in yeast two hybrid strain PJ69-4a. The activity was measured by monitoring *lacZ* expression from *GAL7p* promoter. The values are the averages of the results for three transformants $\pm$ one standard deviation. B. Growth test of PJ69-4a harbouring different GBD-Mxr1 constructs at 30°C on selective amino acid (Trp⁻ and Trp⁻His⁻) and/or amino acid-nucleotide (Trp⁻Ade⁻) deficient plates. C. Indicated Mxr1 fragments encompassing the 246–280 amino acids region having specific Phe to Ala mutation(s) were expressed as GBD (Gal4 DNA binding domain) fusion proteins and activity was measured as described before. Expression level of all of the fusions was also shown in the figure. The values are the averages of the results for three transformants $\pm$ one standard deviation. D. Growth test of PJ69-4a was performed at 30°C as described before.

derepression of *AOX1* expression in ethanol media upon disruption of the interaction with 14-3-3. These observations suggest that the N-terminal 400 amino acids of Mxr1 are sufficient to activate carbon source-dependent expression of Mxr1-dependent genes and provide *in vivo* evidence that the major activation domain identified in *S. cerevisiae* is functional in *P. pastoris*.

Carbon source-dependent phosphorylation of Ser215 and binding of 14-3-3 to Mxr1

The data just presented suggest that phosphorylation-dependent binding of 14-3-3 differentially regulates Mxr1 activity in response to various carbon sources. To examine whether phosphorylation of Ser215 and 14-3-3 binding are

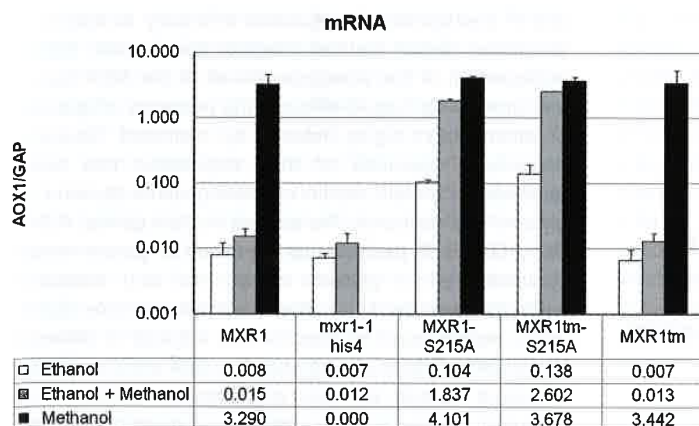


Fig. 7. Studies on expression of major Mxr1-dependent gene, *AOX1* in *P. pastoris* in response to different carbon sources. mRNA was extracted from strains JC100 (*MXR1WT*), JC132 (*mxr1-1 his4*), PPP1 (*MXR1-S215A*), PPP2 (*MXR1tm-S215A*) and PPP3 (*MXR1tm*) both in repressed (0.5% ethanol) and induced (0.5% methanol) as well as ethanol plus methanol growth conditions. Quantitative RT-PCR (qRT-PCR) was performed to determine the levels of *AOX1* mRNA. The mRNA levels were normalized to the levels of *GAP* mRNA in each sample. The error bars represent the mean of three biological replicates assayed in duplicate.

carbon source-regulated we performed Ni^{2+} -NTA and Gst-pulldown assays using *P. pastoris* cell extracts containing C-terminal Myc-(His)₆-tagged truncated Mxr1 (Mxr1tm, 1–400 amino acids) and *E. coli* expressed Gst-C4qzn3. We were unable to detect Mxr1 in the cell extracts either due to its very low level of expression or due to its highly unstable nature. To enrich the protein we performed Ni^{2+} -pulldown using protein extracts from *P. pastoris* strain PPP3 (expressing Mxr1tm-Myc-His₆) after growth in fully repressing medium containing either 0.5% glucose, or 0.5% ethanol; and inducing medium containing 0.5% methanol, PPP2 (expressing Mxr1tm-S215A-Myc-His₆) after growth in 0.5% ethanol containing medium, and JC132, a *mxr1-1* null mutant strain after growth in 0.5% glucose media. Pulldown fractions were immunoblotted with anti-cMyc and anti-pSer230 antibody, as described before. As shown in Fig. 8A Ser215-phosphorylation was diminished significantly in both glucose and methanol growth conditions compared with that observed in ethanol growth condition for wild-type protein. Phosphorylation of the Ser to Ala mutant was also significantly diminished. No protein band was observed corresponding to Mxr1tm for the *mxr1-1* null mutant strain, confirming the specificity of the pulldown assay. A Gst-pulldown was done to test 14-3-3 binding to Mxr1 in different growth conditions using the same cell extracts (except the *mxr1-1* null mutant extract). As shown in Fig. 8B, 14-3-3 binding was also significantly impaired in glucose and methanol grown cells, and completely abolished due to the substitution of Ser to Ala even in ethanol growth media. These results are consistent with the phosphorylation data and together they suggest that Ser215-phosphorylation is regulated by the carbon source available for growth and this in turn regulates 14-3-3 binding to Mxr1. The result in glucose media was quite surprising and interesting. Despite the absence of 14-3-3 binding Mxr1-activated genes are completely repressed, suggesting the presence of a 14-3-3-

independent pathway for the regulation of expression of Mxr1-dependent genes.

14-3-3 acts at a post DNA binding step

The ability of Mxr1 to activate transcription might be regulated at different steps in response to carbon source availability. These include nuclear localization, DNA-binding and post-DNA binding. 14-3-3 could regulate any of these steps. It has been reported that Mxr1 was mostly localized in the cytoplasm in glucose-grown cells and its nuclear localization was increased when cells were grown in ethanol, glycerol, and methanol containing media (Lin-Cereghino *et al.*, 2006). Despite its localization in the nucleus Mxr1 activity remains repressed in glycerol and ethanol media and introduction of a mutation that disrupts its interaction with 14-3-3 partially relieves the repression. These observations prompted us to test whether 14-3-3 inhibits DNA binding by Mxr1 *in vivo*. We performed chromatin immunoprecipitation (ChIP) using cross-linked protein extract containing either C-terminal Myc-tagged truncated wild-type (Mxr1tm) or S215A mutant variant (Mxr1tm-S215A). *P. pastoris* strains PPP2 (expressing Mxr1tm-S215A-Myc) and PPP3 (expressing Mxr1tm-Myc) were grown in 0.5% glucose, 0.5% ethanol and 0.5% methanol-containing media. We tested carbon source-dependent Mxr1-binding to three Mxr1-activated gene promoters, *AOX1prm*, *PEX8prm* and *POX1prm*. As shown in Fig. 9, binding of Mxr1 to these promoters was significantly increased both in ethanol- and methanol-grown cells compared with that observed in glucose grown cells. The binding of Mxr1 to these promoters was consistent with the expression level of the respective genes (Fig. 4A–C). The low level of Mxr1 binding in the presence of glucose could be due to its reduced level of nuclear localization. Interestingly, there was a comparable level of binding of wild-type and mutant protein in both repressing (ethanol) and induc-

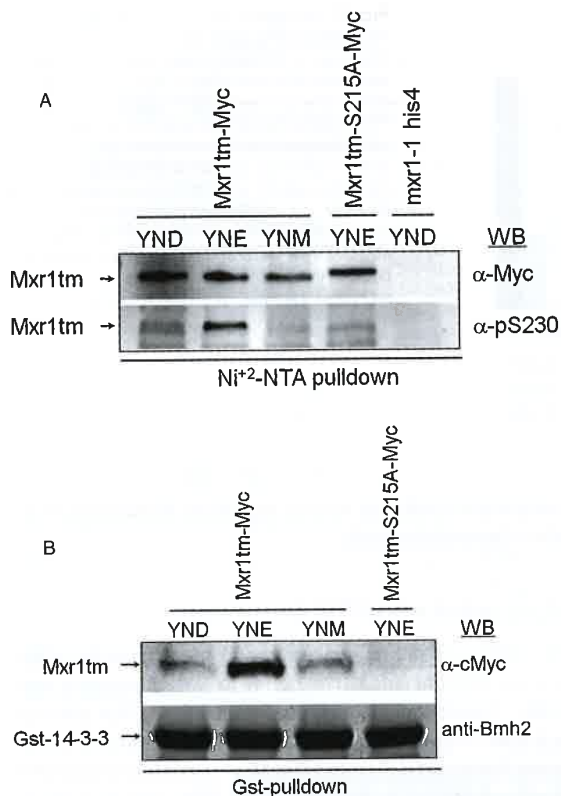


Fig. 8. A. Determination of phosphorylation of Mxr1-Ser215 in response to various carbon sources. Ni^{2+} -pulldown was done using *P. pastoris* protein extract containing either truncated wild-type Mxr1 (Mxr1tm-Myc-His₆) prepared from cells grown in 0.5% glucose (YND), 0.5% ethanol (YNE) and 0.5% methanol (YNM) containing media, or truncated mutant Mxr1 (Mxr1tm-S215A-Myc-His₆) prepared from cells grown in 0.5% ethanol (YNE) containing media, or extract prepared from *mxr1-1* null mutant strain grown in 0.5% glucose containing media (YND). Pulldown fractions were electrophoresed in 4–20% SDS-PAGE and visualized by immunoblotting with anti-cMyc and anti-pSer230 antibody. B. Interaction studies between Mxr1 and 14-3-3 in response to various carbon sources. Gst-pulldown was performed using the *P. pastoris* cell extracts and *E. coli* expressed Gst-C4qzn3 as described above. Pulldown fractions were electrophoresed in 4–20% SDS-PAGE and visualized by Western blotting with anti-cMyc and anti-Bmh2 antibody.

ing (methanol) media, suggests that 14-3-3 does not act to regulate the DNA binding activity of Mxr1, indicating a post-DNA binding role of 14-3-3 in carbon source-dependent regulation of Mxr1 activity. Moreover our results also indicate that besides 14-3-3 other regulatory pathways, are also acting at a post-DNA binding step to regulate Mxr1-dependent gene expression in ethanol.

Discussion

Expression of the methanol utilizing genes among methylotrophic yeasts (*P. pastoris*, *H. polymorpha*, *C. boidinii*

and *P. methanolica*) is regulated differently in response to various carbon sources (Hartner and Glieder, 2006). Independent of the yeast species all of the *MUT* genes are strongly repressed either in the presence of glucose or ethanol and highly induced by methanol. Species-dependent regulation of their expression has been observed in growth media containing either glycerol or glycerol and methanol. The alcohol oxidase genes, *AOX1* and *AOX2* in *P. pastoris* are repressed in growth media containing either glycerol or glycerol and methanol whereas those genes in other methylotrophs are significantly derepressed by glycerol and induced to different levels upon addition of methanol. The differential response to mixed carbon sources suggests the existence of unknown species-specific regulatory machinery(s) for modulating their expression. The zinc-finger transcription factor, Mxr1 is essential for methanol utilization and peroxisome biogenesis in *P. pastoris* (Lin-Cereghino *et al.*, 2006). The molecular mechanism(s) of carbon source-dependent inhibition and methanol-induced activation of Mxr1 is also unknown. Here we report that a *P. pastoris* 14-3-3 protein is involved in this carbon source-dependent regulation.

In the present study we have identified a highly conserved region of Mxr1, residues 212–225, which contains the core 14-3-3 binding region RRASFSA (Parua *et al.*, 2010). As for Adr1, this putative 14-3-3 binding region of Mxr1 differs significantly from mammalian 14-3-3 motifs because it lacks the well-conserved Pro at the +2 position. We report here a previously uncharacterized 14-3-3 family protein in *P. pastoris* that has the ability to complement

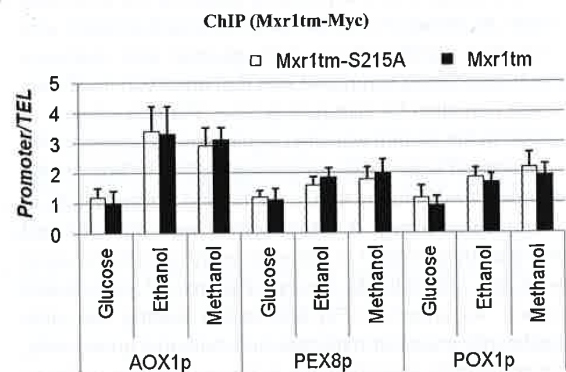


Fig. 9. Chromatin immunoprecipitation. Chromatin immunoprecipitation was done using cross-linked protein extract prepared either from strain PPP2 (Mxr1tm-S215A) or PPP3 (Mxr1tm) after growth in 0.5% glucose, 0.5% ethanol and 0.5% methanol containing media. Immunoprecipitation was done using anti-cMyc antibody against C-terminal Myc-tagged truncated Mxr1 wild-type and mutant protein, Mxr1tm-Myc and Mxr1tm-S215A-Myc respectively. The data are expressed as binding (ChIP/input) for AOX1p, PEX8p and POX1p relative to ChIP/input at the TEL region used as a reference.

S. cerevisiae 14-3-3 functions and to interact with Mxr1 through the 14-3-3-binding motif at residues 212–225 via phosphorylation of Ser215, suggesting that 14-3-3 is a biologically relevant regulator of Mxr1 in *P. pastoris*. Consistent with this interpretation we have observed gene- and carbon source-specific regulation of Mxr1 activity in *P. pastoris* that was dependent on an intact, phosphorylatable 14-3-3 binding motif. Indeed, we observed carbon source-dependent regulation of phosphorylation of Ser215 and consequent carbon source-dependent regulation of 14-3-3 binding to Mxr1, confirming the presence of 14-3-3-mediated regulation of Mxr1 activity in a carbon source-dependent way. Although we observed 14-3-3-mediated inhibition of expression of Mxr1-dependent genes involved in methanol assimilation pathways (*AOX1* and *DHAS*) and peroxisome biogenesis (*PEX8* and *PEX14*) in response to ethanol, there was no 14-3-3-dependent inhibition observed for the genes in glucose- and glycerol-containing media in *P. pastoris*. Ethanol and glucose have been shown to repress the *MUT* pathway genes via two distinct mechanisms (Sakai *et al.*, 1987; Alamae and Liiv, 1998; Parpinello *et al.*, 1998). Indeed, our experimental observations suggest that there is a distinct 14-3-3-independent pathway in glucose-dependent regulation of Mxr1 activity, where nuclear localization could be the predominant pathway that might be regulated by a different mechanism. Based on our present observations we propose that 14-3-3 is a key factor in the mechanism of repression utilized by ethanol, but not by glucose. ChIP results indicate that 14-3-3 might be functioning at a post-DNA binding step, where it could regulate either RNA polymerase II recruitment or pre-initiation complex formation, either by recruiting some repressor or by inhibiting the recruitment of some activating factors. Surprisingly, a different scenario was observed for β -oxidation pathway genes. They were expressed constitutively in ethanol medium essentially through an Mxr1-dependent pathway, although at lower levels than that observed in methanol. 14-3-3-mediated regulation of their expression was also observed in glucose and glycerol. This suggests that the Mxr1-dependent activation mechanism for expression of β -oxidation pathway genes is distinct from that of the *MUT* pathway genes. We propose that Mxr1 might be acting through and/or in conjunction with the Pip2/Oaf1 homologues of *P. pastoris* to regulate the expression of β -oxidation pathway genes as has been observed for Adr1 in *S. cerevisiae* (Gurvitz *et al.*, 2000; 2001; Biddick *et al.*, 2008; Ratnakumar and Young, 2010).

We have mapped the location of the major activation domain of Mxr1 to residues 246–280. We have confirmed this mapping in *P. pastoris* by showing that full activity resides within the first 400 amino acids of Mxr1, which includes the N-terminally located DNA binding domain at residues 29–101. The amino acid sequence alignment in

Fig. 1 shows that the C-terminal end of the Mxr1 activation domain is in a position analogous to the location of the cryptic activation domain of Adr1 at residues 260–360 (Cook *et al.*, 1994; Parua *et al.*, 2010). Although the two amino acid sequences show no significant overall homology, they are both enriched in hydrophobic and acidic residues, a characteristic of canonical activation domains. Indeed, our mutational analyses indicate that the Phe residues at positions 249, 254 and 278 are important for Mxr1 activity. This is consistent with reports that hydrophobic residues are important for the activity of a number of other activation domains (Cress and Triezenberg, 1991; Regier *et al.*, 1993; Blair *et al.*, 1994; Gill *et al.*, 1994; Lin *et al.*, 1994; Drysdale *et al.*, 1995; Folkers *et al.*, 1995; Almlöf *et al.*, 1997), including the major activation domain of Adr1 (Young *et al.*, 1998) located at amino acids 420–462 (Cook *et al.*, 1994; Young *et al.*, 2002). The presence of the major activation domain of Mxr1 at a location analogous to the cryptic activation domain of Adr1 suggests that either Mxr1 has lost a second activation domain analogous to the Adr1 major activation domain or Adr1 has gained this domain.

Despite the presence of remarkable N-terminal sequence similarities between *S. cerevisiae* Adr1 and *P. pastoris* Mxr1, these proteins share no other regions of significant sequence similarity. Both proteins have similar but not identical regulatory features. Adr1 activates genes that are important for growth on non-fermentable carbon sources, for peroxisome biogenesis, and for β -oxidation of fatty acids (Ciriacy, 1979; Simon *et al.*, 1992; 1995; Sloan *et al.*, 1999; Young *et al.*, 2003). Mxr1 activates genes responsible for methanol utilization, peroxisome biogenesis (Lin-Cereghino *et al.*, 2006) and our present study suggests its involvement in the activation of β -oxidation pathway genes. A major difference in the gene regulation in which these factors participate is that distinct methanol-mediated induction and derepression mechanisms exist for Mxr1-regulated genes, while only derepression in the absence of induction has been reported for Adr1-dependent genes. The canonical Mxr1-regulated genes, *AOX1* and *DHAS*, are repressed tightly by glucose, glycerol and ethanol, and highly induced by methanol in the absence of another carbon source (Ellis *et al.*, 1985; Cregg *et al.*, 1989; Koutz *et al.*, 1989; Lin-Cereghino *et al.*, 2006), while repression of the canonical Adr1-dependent gene, *ADH2*, is lifted by shifting the cells to growth on glycerol, ethanol or other non-fermentable carbon sources or upon glucose starvation (Ciriacy, 1975; Denis, 1987; Young *et al.*, 2003). Our studies suggest that phosphorylation-dependent 14-3-3 binding to a domain located between the DNA-binding domain and the major activation domain of Mxr1 and Adr1 plays an important role in regulating the activity of both transcription factors. This suggests that carbon source- and 14-3-3-dependent regulation of tran-

scription factor activation domain function is conserved in fermentative yeast like *S. cerevisiae* and *P. pastoris* and may be present in other species containing analogous transcription factors. It will be interesting to determine whether 14-3-3 in the divergent yeasts regulates the activities of these transcription factors in a similar manner despite their lack of overall sequence conservation.

Experimental procedures

Yeast strains and growth of cultures

The *S. cerevisiae* strains used in this study are listed in Table 1. *S. cerevisiae* cultures were grown in either yeast extract peptone medium or in synthetic medium containing 2–5% glucose and lacking the appropriate amino acid or nucleotide for plasmid selection. To maintain selection for plasmids containing *TRP1* and/or *URA3* the synthetic selective medium contained 0.2% casamino acids rather than the standard dropout solution. All *S. cerevisiae* strains were grown at 30°C unless otherwise mentioned.

The *P. pastoris* strains used in this study are listed in Table 1. *P. pastoris* cells were cultured in either YPD medium (1.0% yeast extract, 2.0% peptone, 2.0% glucose) or minimal YNB medium (0.17% yeast nitrogen base without amino acids and 0.5% ammonium sulphate) supplemented with either 0.5% glucose (YND), 0.5% methanol (YNM), 0.5% glycerol (YNG) or 0.5% ethanol (YNE). Amino acids were added to 50 µg ml⁻¹ when required. *P. pastoris* strains were grown at 30°C and transformations were done by using electro-transformation (Cregg and Russell, 1998).

Escherichia coli cells, mainly DH10β and BL21 (DE3), were cultured in Luria–Bertani medium at 37°C for recombinant DNA techniques and protein overexpression.

Gene expression studies on different carbon sources

For gene expression studies in response to different carbon sources, cells were first grown in YND medium to an optical density at 600 nm (A_{600}) of approximately 0.5. These glucose-grown cells were centrifuged, washed with water and resuspended either in pre-warmed YNM, YNE, YNG or YND supplemented with any other necessary ingredients. The cells were grown with vigorous shaking at 30°C for 8–10 h, and harvested by centrifugation and processed for RNA extraction.

Plasmid constructs

All *MXR1* constructs were made by cloning the corresponding coding sequence into the *TRP1-CEN4* vector, pOBD2 using gap repair methods (Orr-Weaver and Szostak, 1983; Szostak *et al.*, 1983), where proteins were expressed as N-terminal GBD (Gal4 DNA binding domain)-fusion as described in Parua *et al.* (2010). PCR fragments representing various regions of *MXR1* were generated using forward and reverse primers that contained homology to the vector sequences flanking the poly-linker region of pOBD2 as well as homology to *MXR1*. The *NcoI* and *PvuII*-digested pOBD2 plasmid (Uetz *et al.*, 2000) and a PCR fragment were used to transform PJ69-4a to

Trp⁺ prototrophy. Plasmid DNA from two or three Trp⁺ transformants was rescued and sequenced to confirm that recombination had produced the correct in-frame gene fusion using primers, OBDsF and OBDsR (see Table S1). Western analysis with an anti-Gal4 DBD monoclonal antibody (RK5C1, Santa Cruz Biotechnology) was used to confirm the synthesis of a fusion protein of the correct size. The primers used are listed in Table S1.

pGEX-3X-*C4QZN3* and pBF-*C4QZN3* were made by PCR amplifying *C4qzn3* (*Pichia pastoris* 14-3-3) coding sequence from *P. pastoris* genomic DNA using *C4QZN3_BamHI_F* and *C4QZN3_BamHI_R* primers (see Table S1). BamHI-digested PCR fragment was then cloned into the BamHI site of pGEX-3X (GE Healthcare Life Sciences) to produce pGEX-3X-*C4QZN3*. pBF-*C4QZN3* was generated by replacing *BMH1* from pBF-*BMH1* [a gift from S. Zheng; (Bertram *et al.*, 1998)] with the above mentioned BamHI digested PCR product. Positive clones were checked by restriction digestion with BamHI and *EcoRI* and the sequence and directionality of the insert was tested by DNA sequencing analysis using sequencing primer GEX_sF for pGEX-3X-*C4QZN3* and BF339_sF for pBF-*C4QZN3* (listed in Table S1).

All the plasmids used in this study are listed in Table S2.

Western blot

Western blot analyses were performed according to the manufacturer's instructions for the Odyssey infrared imaging system (Licor Biosciences, Lincoln, NE), using 1:500 to 1:1000 diluted monoclonal anti-GBD (Santa Cruz Biotechnology, RK5C1) or anti-cMyc (Santa Cruz Biotechnology, 9E10) or polyclonal anti-Bmh2 (a gift from S. Lemmon) or anti-pSer230 (custom raised against Ser230-phosphorylated *Adr1* sequence from BETHYL Laboratories) antibody as primary antibodies and Licor λ_{800} secondary antibodies.

Preparation of protein extracts from yeast cells

Protein extracts from yeast cells were prepared following the procedure as described in Parua *et al.* (2010). Typically 50–100 ml of yeast cell culture grown to an A_{600} of ~1 was used for protein extraction. Cells were collected by centrifugation at 1600 *g* for 5 min at 4°C in a Sorvall RC3B-plus centrifuge, washed once with 15% glycerol containing 1 mM phenylmethylsulphonyl fluoride (PMSF), and resuspended in an equal volume of ChIP lysis buffer (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) containing protease (Sigma) and phosphatase inhibitors (Roche Applied Science). The cells were broken with glass beads in a Savant FP120 FastPrep machine with two disruption cycles of 45 sec at a speed setting of 4.0. The unbroken cells and debris were pelleted by centrifugation in a microcentrifuge at 13 600 *g* for 10 min. The clarified extract was collected in a fresh microcentrifuge tube containing 1 mM PMSF and 1× phosphatase inhibitor and used in subsequent experiments.

GST-pulldown assays

The Gst-pulldown assays were done following the same protocol essentially as described in Parua *et al.* (2010). GST-

Bmh1 and *P. pastoris* Gst-14-3-3 fusion proteins were expressed from pGEX-3X-BMH1 and pGEX-3X-C4QZN3, respectively in *E. coli* BL21 (DE3). These fusion proteins were then immobilized on glutathione-sepharose 4B beads as described by the manufacturer (GE Healthcare Life Sciences). Pulldown assays were performed using 30–40 µg of sepharose 4B coupled Gst-fused proteins and yeast extract containing ~2 mg of total proteins in 1× PBST (Phosphate buffered saline containing 0.1 % Tween 40), 1× protease inhibitor and 1× phosphatase inhibitor. The suspension was incubated at 4°C for one hour with continuous nutation. The beads were then pelleted by centrifugation at 320 *g* for 1 min, washed three times with 1× PBST and re-suspended in 60 µl of 2× LDS-NuPAGE sample buffer followed by boiling at 95°C for 5 min. Fractions collected at different steps of the pulldown assays were then analysed by SDS-PAGE and Western blotting.

Ni²⁺-NTA pulldown assays

Ni²⁺-NTA pulldown assays were done using *P. pastoris* cell extract expressing C-terminal His₆-tagged truncated wild-type or S215A Mxr1 (encodes 1–400 amino acids) mutant. Yeast extract containing approximately 10 mg total protein was incubated with Ni²⁺-NTA beads at 4°C for one hour with continuous nutation in binding buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5% glycerol and 10 mM imidazole). The beads were then pelleted by centrifugation at 320 *g* for 1 min, washed three times with wash buffer (same as binding buffer except 25 mM imidazole) and re-suspended in 2× LDS-NuPAGE sample buffer followed by boiling at 95°C for 5 min. Pulldown fractions collected were then analysed by SDS-PAGE and Western blotting.

mRNA isolation and qRT-PCR

mRNA was isolated from yeast strains grown in either repressing or inducing medium using the acid phenol method described in Collart and Oliviero (2001). Residual DNA in the RNA preparation was reduced by treatment with DNase I (Ambion) following the manufacturer's recommendations. cDNA was synthesized using SuperScript III (Invitrogen) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) was performed for measuring mRNA levels using diluted cDNA. A standard curve was generated with *GAP* primers and used to quantify the mRNA levels. Samples were prepared from biological triplicates and analysed in duplicate.

β-Galactosidase assays

β-Galactosidase assays were performed as described by Guarente (1983) after growing the cells at 30°C in selective medium containing 2–5% glucose to an A₆₀₀ of approximately 1. The reported values in Miller units are the averages of three transformants.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed as described in Biddick *et al.* (2008). In brief, cells from a 50 ml

culture at an A₆₀₀ of ~1 were pelleted at 1600 *g* at room temperature in a Sorvall RC3B-plus centrifuge and resuspended in 8.75 ml of phosphate-buffered saline (PBS). Ethylene glycol-bis (succinimidylsuccinate) (EGS; Thermo Scientific; 21565) cross-linker dissolved in DMSO was added to a final concentration of 3 mM and the sample was swirled at room temperature for 45 min. The EGS cross-linked cells were pelleted at 1600 *g* at room temperature and resuspended in 25 ml of 1× PBS supplemented with 1% formaldehyde. After shaking gently for 15 min at room temperature, 1.5 ml of 2.5 M glycine was added and the cells were pelleted. The cell pellet was washed once with 10 ml of tris-buffered saline (TBS) containing 125 mM glycine and once with 10 ml of TBS only. Protein extract was prepared as described above after resuspending the cells into ChIP lysis buffer containing protease and phosphatase inhibitor cocktail and the clarified extract was then used in subsequent immunoprecipitation (IP) experiments. IP of ~6 mg of protein extract (Tachibana *et al.*, 2007) was performed overnight with constant nutation at 4°C with anti-cMyc (9E10; Santa Cruz Sc40). After IP, 50 µl of Protein A-coated Mag Sepharose™ Xtra beads from GE Healthcare (Cat. No. 28-9670-62) were added and nutation was continued for 1–2 h at 4°C. After separating the beads, 10 µl of the supernatant was aliquot out and mixed with 140 µl of ChIP elution buffer (50 mM Tris-HCl, pH 8.0, 1% SDS, 1 mM EDTA) for measuring the amount of input DNA. The beads were processed by washing twice with ChIP lysis buffer, twice with high salt ChIP lysis buffer (ChIP lysis buffer with 0.5 M NaCl), twice with ChIP wash buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% Nonidet-P40, 0.5% sodium deoxycholate), and twice with ChIP TE (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA). The immunoprecipitated DNA bound to the beads was eluted at 65°C for 10 min with 150 µl elution buffer. The eluted and input DNA were incubated for 12–16 h at 65°C and then purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol. The eluted and input DNA were diluted 20- and 100-fold, respectively, and quantification of specific sequences was performed by qPCR using Power SYBR Green master mix (Applied Biosystems) in a PTC-200 Thermocycler coupled to a Chromo 4 continuous fluorescence detector (MJ-Research). Opticon 3 software (MJ-Research) was used for the data analysis. Occupancy of a protein is expressed as fold-increase of the IP to input ratio of the amount of the specific amplicon for the gene sequence over the IP to input ratio corresponding to the amplicon for the telomeric sequence. Primers used for ChIP analysis are listed in Table S1.

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Review

Recombinants proteins for industrial uses: utilization of *Pichia pastoris* expression system

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Abstract

The innovation in industrial process with impact in the efficient production is the major challenge for actual industry. A high numerous of enzymes are utilized in at different level of process; the search for new alternatives with better characteristic has become a field of study of great interest, the recombinant protein achievement in a different host system is an alternative widely assessed for production of this. The microorganism *Pichia pastoris* has been used like a successful expression system in diverse areas, improved the yield and extraction-recovery of the product expressed. The reported of diverse authors in the production of enzymes with different application in industry is varied, in this review the different industry areas and the characteristic of the enzymes produced are detailed.

Key words: recombinant protein, protein production, yeast, industrial applications.

Recombinant Protein for Industrial Use

The production of protein for industrial use started in the end of century XIX, the industry searched new component for improve the efficiency of the process or searching alternatives to make it a most cost effective process; the development of recombinant DNA technology and the progress made in the optimization of the bioprocess with recombinant organisms offers a wide variety of alternatives in the production of proteins for use in industrial processes with new and/or better properties.

The expression systems based of recombinant proteins in yeast have proven to be an efficient and economical source of proteins of industrial interest higher eukaryotes and / or academic (Buckholz and Gleeson, 1991) becoming one of the most frequently used alternative for the production of proteins a high scale.

Pichia pastoris Like an Expression System

The use of the methylotrophic yeast, *Pichia pastoris*, as a cellular host for the expression of recombinant proteins

has become increasing popular in recent times. *P. pastoris* is easier to genetically manipulate and culture than mammalian cells and can be grown to high cell densities. Equally important, *P. pastoris* is also a eukaryote microorganism, and thereby provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications required for functionality. Additionally, linearized foreign DNA can be inserted in high efficiency via homologous recombination procedures to generate stable cell lines whilst expression vectors can be readily prepared that allow multiple copies of the target protein, multimeric proteins with different subunit structures, or alternatively the target protein and its cognate binding partners, to be expressed (Daly *et al.*, 2004).

Recombinant protein production in the yeast strain *Pichia pastoris* has several advantages over other eukaryotic and prokaryotic expression systems: (i) rapid growth rate, coupled with ease of high cell-density fermentation; (ii) high levels of productivity in an almost protein-free medium; (iii) elimination of endotoxin and bacteriophage con-

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tamination; (iv) ease of genetic manipulation of well-characterized yeast expression vectors; (v) absence of known human pathogenicity in the spectrum of lytic viruses that prey on *P. pastoris*; (vi) diverse post-translational modifications that include polypeptide folding, glycosylation, methylation, acylation, proteolytic adjustment, and targeting to subcellular compartments; and (vii) the ability to engineer secreted proteins that can be purified from growth medium without harvesting the yeast cells themselves (Li *et al.*, 2007).

Pichia pastoris has been exploited by biotechnologists for the high-level production of foreign proteins (Cregg *et al.*, 2004; Romanos *et al.*, 1992). In this review will focus in the different recombinant proteins with application in diverse industrial areas expressed in the system *P. pastoris*, highlighting the applicability and the main improvements obtained through this expression system.

Animal Feed Additive

Monogastric animals are unable to utilize phytic acids due to the low levels of phytase activity in their digestive tracts, by that an inorganic phosphate source is commonly added into the feed for the purposes of phosphorous supplementation (Torre *et al.*, 1991). The enzyme phytase catalyze the hydrolysis of phytic acid (*myo*inositol hexakisphosphate), the major storage form of phosphorous in plant seeds (Cheryan, 1980) thereby releasing inorganic phosphate, is regarded as an antinutrient factor since it forms insoluble complexes with proteins and a variety of nutritionally important metal ions such as calcium, zinc, magnesium, and iron, and decreasing the bioavailability of phosphorous (Torre *et al.*, 1991). Microbial phytases available in the market have problem in thermostability and degradation suffers in stomach digestion; becoming not feasible for addition in feed rations like additive. An alternative arises from *P. pastoris* for obtaining protein from prokaryotes or eukaryotes microorganisms and with high yield of enzyme.

Phytase from *Aspergillus fumigates*

In the investigation realized by Rodriguez *et al.* (Rodriguez *et al.*, 2000) the analysis was focused on the differences presented in the recombinant protein obtained from *Pichia pastoris* vs. those obtained in other expression systems. The gene r-Afp expressed in *P. pastoris* in pPICZαA vector, obtained yield of 729 mg of purified protein per liter of culture, with a specific activity after purification of 43 U/mg at pH 5.5 of protein has a comparatively higher specific phytase activity than those reported values of the enzyme produced by *A. niger*, *H. polymorpha*, and *S. cerevisiae* which varied between 23.4 to 28.1 U/mg protein. The remarkable resistance of the *A. fumigates* phytase to heat inactivation is most appealing characteristic (Pasa-montes *et al.*, 1997). In assay for temperature stability was conserved at 65 °C around of 57% and for 90 °C around of

40%. Another interesting observation in this study is the relatively high resistance of r-Afp to pepsin. Given the ratios of pepsin/phytase (w/w) from 0.001 to 0.1, r-Afp remained essentially intact after 2 h of incubation at 37 °C. On the other hand, the enzyme was degradable by trypsin depending on the ratios. While r-Afp was partially digested from the ratio of 0.001 to 0.02, the enzyme was fully degraded at the ratio of 0.1; suggests this results that r-Afp has different susceptibilities to proteolysis of pepsin and trypsin.

Phytase from *Escherichia Coli*

In this study executed by Chen *et al.* (2004) was evaluated and compared the production of recombinant protein in flask scale and high cell-density fermentation, the *appA* gene cloned in pPICZαA vector of *P. pastoris* registered phytase activity level was enhanced from 118 to 204 U/mL at the flask scale and 1880-4946 U/mL for high cell-density fermentation, this high yield would thus suggest that phytase production by *P. pastoris* was both economical and feasible. The most important result in this study is focusing in modified media for enhances the induction of recombinant protein; the phytase production was increased when a modified mBMMHY medium was used. The mBMMHY medium was modified from BMMY medium by replacing peptone and YNB with histidine and the YE concentration was reduced from 1 to 0.1%. The phytase activity in mBMMHY was 204 U/mL after an induction period of 96 h, virtually twice the level that it achieved when induction was conducted in BMMY or FBSH 118 and 123 U/mL respectively after 96 h induction. The protein concentration of cells induced in mBMMHY medium reached 6.4 g/L of culture supernatant and the phytase activity was noted to be 4946 U/mL after an induction period of 192 h.

Gelatin industry

Gelatin is in essence, denatured collagen and is prepared by hot acid or alkaline extraction of animal tissues such as bones and hides; is a well-known biopolymer and has a long history of use, mainly as a gelling agent in food. Certain characteristics limited their applications, the extraction procedure results in chemically modified gelatin peptides covering a wide range of molecular weights (Asghar and Henrickson, 1982) and traditional gelatin has a high gelling temperature due to its high content of helix-stabilizing hydroxyprolines. This can be undesirable for low temperature applications (Saddler and Horsey, 1987).

Production of non-gelling (*i.e.* non-hydroxylated) gelatin-like proteins in *Escherichia coli* has been reported. It involves the expression of synthetic genes constructed from repeating Gly-Xaa-Yaa encoding units (Gardner *et al.*, 1992). Many problems arise concerning the instability of these highly repetitive genes (Capello, 1990). Probably, native gelatin sequences derived from collagen genes are more stable than synthetic gelatin-like sequences, because

there is greater variation in amino acid usage and less repetitiveness (Fahnestock and Bedzyk, 1997).

Non-gellins gelatin

In report, generated by Werten *et al.* (1999) the production of high extracellular non-gelling gelatin in pPIC9 and a multicopy pPIC9K expression vectors of *Pichia pastoris* was described. A 0.7 kb rat denominated *COL3A1* cDNA fragment, encoding a 21 kDa gelatin and a 1.0 kb mouse denominated *COL1A1* cDNA fragment encoding a 28 kDa gelatin (Colla1-1) and a 1.8 kb mouse denominated *COL1A1* cDNA fragment encoding a 53 kDa gelatin (Colla1-2) were cloned. Different strategies were used to reduce the proteolytic degradation of this extremely vulnerable unfolded protein, the best results was obtained from a transformant bearing approximately 15 copies of the pCOL3A1 vector produced up to 14.8 g gelatin/liter extracellular medium and the problems of proteolytic degradation could be minimized despite the unfolded structure of gelatin through modifications of pH fermentation and supplement the fermentation medium with casamino acids; proteolytic cleavage at specific mono-arginylic sites, by a putative Kex2-like protease, could be

successfully abolished by site-directed mutagenesis of these sites.

Wastewater Treatment and Bleaching

Laccases are blue copper-containing phenol oxidases that are widely distributed in plants and certain fungi (Alcalde, 2007). Have been ascribed diverse biological functions in different organism; the potential applications include (i) delignification and biobleaching of pulp (Bourbonnais *et al.*, 1997) (ii) treatment of wastewater from industrial plants (Bergbauer *et al.*, 1991) (iii) enzymatic modification of fibers and dye-bleaching in the textile and dye industries (Abadulla *et al.*, 2000). A possible advantage with *P. pastoris* compared to many filamentous fungi is that it does not produce cellulolytic enzymes and laccase produced in this host could, therefore, potentially be applied directly in the pulp and paper industry without any purification.

Laccase from *Trametes versicolor*

The work realized by Hong *et al.* (Hong *et al.*, 2002) utilized the gene laccase *lcl1* and was cloned into *P. pastoris* in pHIL-D2 expression vector. Was employed the BMM medium, and the highest laccase activity registered was 3.3 U/mL, which was observed after 3 days of induction; after that it declined to 1.6 U/mL. The decrease in laccase activity during the late period in shake-flask cultivation might be due to the low pH. The temperature is an important parameter for optimization of laccase expression in yeast systems (Cassland and Jonsson, 1999), the results confirmed that cultivation at low temperature in shake-flask improved laccase activity 3.3 U/mL at 30 °C and

11.5 U/mL at 20 °C. When fermentor cultivations was used the pH was kept at 5.0 and in the protease assay higher proteolytic activity was found at pH 5.0 than at pH 8.5, a previous investigation indicated that proteolytic activity played an important role in laccase expression by *P. pastoris* because the protease deficient SMD 1168 strain performed better than GS115. In the study a positive correlation was established between the amount of methanol consumed and the quantity of protein produced was observed, however a fivefold higher volumetric laccase activity was obtained when the methanol concentration was kept at 0.5% instead of 1.0%.

Laccase from *Pleurotus sajor-caju*

Research realized by Soden *et al.* (2002) report the cloning of the full-length gene for one of these isozyme genes, namely *Psc lac4*, and on its subsequent expression in the heterologous host *P. pastoris*. The GS115 strain was utilized transformed with pPIC3.5-*lac4*, the level of *Psc Lac4* expression reported here was 4 ± 85 mg/L the purified protein had an activity of 1050 U/mL, with a specific activity of 2100 units mg^{-1} . *Psc Lac 4* was stable for at least 5 h between pH 6 and pH 8. Over the same period of time, the activity decreased to approximately 80%, 70% and 64% of the original value when the enzyme was incubated at pH 5, pH 3 and pH 9, respectively. At 25 °C, the pH stability of *Psc Lac 4* was reduced. The optimal temperature for *Psc Lac4* activity was 35 °C with ABTS as substrate. *Psc Lac4* was relatively stable after 1 h at 35 °C; however, over the same period of time residual activity decreased to approximately 35% and 25% of the original value when incubated at 35 °C and 45 °C respectively, while no detectable activity was observed after 1 h at 65 °C.

Laccase from *Pycnoporus cinnabarinus* I-937

Studies realized by Otterbein *et al.* (2000) was dedicated to isolation and characterization of a cDNA corresponding to the gene *lac1* isolated from fungus *P. cinnabarinus* I-937 and its expression in the methylotrophic yeast *P. pastoris*; two distinct expression plasmids were used. Plasmid pPICZB/*Lac1* and pPICZaB/*Lac1* differs from the first in that it contains the native *S. cerevisiae* α -factor secretion signal upstream of the sequence of mature laccase. Expression was evaluated in baffled flasks and for pPICZaB/*Lac1* construction, the laccase activity gradually reached 20 units, and the cell culture density reached a D600 of 21 after 10 days of incubation for other hand for the pPICZ/*Lac1* expression vector, laccase activity reached a maximum of 12 units and the cellular density reached a D600 value of 17 after 8 days, the expression level was satisfactory 8 mg/L with the two expression vectors used, but the eventual proteolytic degradation events in the culture medium could not be regulated, the regulation of pH in the media utilized was important factor for the production of

laccase enzyme, obtaining in acidic pH an detrimental effect in the production.

Antifreeze

Antifreeze proteins (AFP) have been isolated from a number of organisms including fish, insects, plants and bacteria (Davis and Hew, 1990). Their properties include depressing freezing temperature without affecting the melting temperature (so-called thermal hysteresis), modifying ice crystal morphology, inhibiting ice crystal growth (recrystallization), enhancing cellular integrity, and reducing microbial growth (Chapsky and Rubinsky, 1997; Harding *et al.*, 1999).

The use of AFPs is focused on industry foodstuffs that involved frozen storage. However, according to their properties, AFPs are likely to be good preservatives, not only for frozen food but also for chilled food (Griffith and Ewart, 1995).

In the work realized by Loewen *et al.* (1997) describing the shake-flask expression of the cystine-rich SRAFP in *Pichia* obtaining secreted recombinant protein up to 5 mg/L and demonstrate that the recombinant protein has identical activity to SRAFP isolated from sea raven serum. In order to increase yields further, four different strategies were tested in 10 liter fermentation vessels, the mixed-feeding/Mut^s strategy proved to be the most efficient with SRAFP yields reaching 30 mg/L.

Lytic Enzymes

Enzymes such as cellulases, xylanases, proteases, lipases, amylases, phosphatases and pectinases are widely used in the pulp and paper, textiles, detergent, food and beverage and pharmaceutical industries (Cheng *et al.*, 1999).

The main industrial application of pectinases is the extraction and clarification of fruit and vegetable juices. Most of the microbial pectinases produced by the industry are dedicated to this purpose. Pectins are responsible for the turbidity and consistency of the juice causing an increase in their viscosity, which hinders its clarification, filtration and concentration (Alkorta *et al.*, 1998).

Polygalacturonases are the pectic enzymes which are most commonly used in the case of orange juice, where natural pectin esterases are present, pectin is only partially methylated. In the process of orange juice extraction, pectinases can be added at the end of the pulp wash extraction to reduce viscosity or, preferably, at the end of the first finisher. This leads to higher yield in juice, a better extraction of soluble solids and to a lower viscosity. The action of these enzymes just reduces the viscosity without attacking the insoluble pectin that maintains the stability of the cloud (Kashyap *et al.*, 2001). The endopolygalacturonase is the most substantial member of pectolytic enzymes that are uti-

lized to degrade various pectic substances (Whitaker, 1990).

Cellulases and xylanases are active fibrolytic enzymes and have sparked interest for a number of biotechnological applications. These include development of probiotic and as feed additives for silages and total mixed rations, for saccharification of lignocellulosic residues and for production of polysaccharide-hydrolysing enzymes (Li *et al.*, 1996).

Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in plant cell walls. Xylanolytic enzymes are mainly produced by saprophytic fungi and bacteria and hydrolyze the xylan component of plant cell walls (Coughlan and Hazlewood, 1993).

Endopolygalacturonase from *Botrytis cinerea*

In investigation realized by Kars *et al.* (2005) the coding sequences of the genes Bcpg1, Bcpg2, Bcpg3, Bcpg4 and Bcpg6 were cloned in the expression vector pPIC3.5 of *P. pastoris*. Production levels of BcPGs were reproducible for individual enzymes but varied significantly between the different enzymes, ranging from 5 mg/L for BcPG1 to 1000 mg/L for BcPG3. The overall yield of pure protein of the different BcPGs was highly variable ranged from 0.5 to 500 mg per fermentation. The optimal enzyme activity using polygalacturonic acid (PGA) as substrate was observed at pH 4.2 for BcPG1, pH 4.9 for BcPG4, and pH 4.5 for BcPG2 and BcPG6. Purified BcPG3 displayed a broader pH optimum ranging from pH 3.2 to 4.5.

Endo- β -1,4-xylanase from *Aspergillus niger*

In the working realized by Berrin *et al.* (2000); the cDNA-encoding XylA was used to transform *P. pastoris* and the cells with construction obtain a secretion yields up to 50-100 mg/liter were obtained in selective medium. The specific activities of recombinant xylanase of *A. niger* were 350 ± 25 U mg/L for the one expressed in *Pichia* and 270 ± 16 U mg/L for the native xylanase over expressed. The optimum reaction temperature of both xylanases was found at 50 °C and over 70% of maximal activity between 40 and 60 °C. Around 90% activity remained after 3 h incubation at 50 °C but between 3 and 4 h of incubation at this temperature, xylanase activity dropped dramatically and decreased by more than 50%.

Conclusion

All revised investigation in this review demonstrating the major production of product when the *Pichia pastoris* expression system was utilized and when this is compared with other expression system like *E. coli*, *S. cerevisiae* or *H. polymorpha*. Recombinant protein obtained in *P. pastoris* system exhibit one aspect that has improved is the specific activity of the enzyme produced, detecting in-

creased enzyme activity when this was assessed. Being this a highly relevant aspect because when a recombinant protein is destined to industry use, the yield of the product is an important factor for profitability of the process.

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Review

Biotechnology and genetic engineering in the new drug development. Part I. DNA technology and recombinant proteins

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Abstract:

Pharmaceutical biotechnology has a long tradition and is rooted in the last century, first exemplified by penicillin and streptomycin as low molecular weight biosynthetic compounds. Today, pharmaceutical biotechnology still has its fundamentals in fermentation and bioprocessing, but the paradigmatic change affected by biotechnology and pharmaceutical sciences has led to an updated definition. The biotechnology revolution redrew the research, development, production and even marketing processes of drugs. Powerful new instruments and biotechnology related scientific disciplines (genomics, proteomics) make it possible to examine and exploit the behavior of proteins and molecules.

Recombinant DNA (rDNA) technologies (genetic, protein, and metabolic engineering) allow the production of a wide range of peptides, proteins, and biochemicals from naturally nonproducing cells. This technology, now approximately 25 years old, is becoming one of the most important technologies developed in the 20th century.

Pharmaceutical products and industrial enzymes were the first biotech products on the world market made by means of rDNA. Despite important advances regarding rDNA applications in mammalian cells, yeasts still represent attractive hosts for the production of heterologous proteins. In this review we describe these processes.

Key words:

biotechnology, recombinant DNA technology, directed mutagenesis, aranesp, activase

Introduction

Biotechnology is being defined as organisms or biological molecules, which are used in the industrial production. This term includes processes used for

centuries, such as the production of alcohol, as well as the discovery in more recent years of genetic engineering [30, 40, 46]. Biotechnology in the pharmacology is being called biotechnology pharmaceutical, which is producing biopharmaceuticals. These are proteins with therapeutic meaning and recently nu-

cleic acid used in the gene therapy, which are being produced due to genetic engineering or traditional biotechnology.

The biotechnology industry has been developing most dynamically for the last 30 years of the 20th century. Today, there exist over 10,000 pharmaceutical companies producing in total about 5000 biopharmaceuticals. However, only 100 of these companies have a prominence and meaning in the industry. The medical success of sulfonamides and insulin gave new emphasis to the industry, which was boosted further by the commencement of industrial-scale penicillin manufacture in the early 1940s [32, 46, 48].

In the years 1971–1973 a new technology was brought into effect, which then became a great scientific turning point. It was recombinant DNA technology, otherwise known as genetic engineering, which until today is a base of many biotechnological processes. PCR (Polymerase Chain Reaction) is one of elements of the technology behind recombined DNA. The technique discovered in 1985 by Mullis enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. This automated process bypasses the need to use bacteria for amplifying DNA [11].

The basic biotechnological processes used most widely in the pharmaceutical industry apart from recombined DNA technology, and including within this directed mutagenesis, are biocatalysts, technology of monoclonal antibodies, technology of vaccines, metabolic engineering, and only recently also gene therapy. However, these processes are largely based on the tools of gene engineering. Biotechnology has also had a major impact on the pharmaceutical industry.

Recombinant DNA technology

The idea for recombinant DNA was first proposed by Peter Lobban, a graduate student of Prof. Dale Kaiser in the Biochemistry Department at Stanford University Medical School. Recombinant DNA technology is the technique, which allows DNA to be produced *via* artificial means. The procedure has been used to change DNA in living organisms and may have even more practical uses in the future. It is an area of medicine, which is at present in its initial phase of the overall concerted effort [32].

The cornerstone of most molecular biology technologies is the gene. To facilitate the study of genes, they can be isolated and amplified. One method of isolation and amplification of a gene of interest is to clone the gene by inserting it into another DNA molecule that serves as a vehicle or vector that can be replicated in living cells. When these two DNAs of different origin are combined, the result is a recombinant DNA molecule [39].

Currently, recombinant DNA technology has attracted headlines when it has been used on animals, either to create identical copies of the same animal or to create entirely new species. One of these new species is the GloFishTM, a type of fish that seems to glow with a bright fluorescent coloring. While they have become a popular aquarium fish, they have other uses as well. Scientists hope to use them to help detect polluted waterways, for example.

Recombinant DNA technology is not accepted in certain quarters, especially social conservatives, who feel the technology is a slippery slope to devaluing the uniqueness of life. Furthermore, because some DNA work involves the use and destruction of embryos, there is more controversy created. Still, proponents of the technology say the ultimate goal is to benefit human life, not destroy it.

How to obtain genes?

Two major categories of enzymes are important tools in the isolation of DNA and the preparation of recombinant DNA: restriction endonucleases and DNA ligases. Restriction enzymes are DNA-cutting enzymes found in bacteria. Because they cut within the molecule, they are often called restriction endonucleases. In order to be able to sequence DNA, it is first necessary to cut it into smaller fragments. Many DNA-digesting enzymes can do this, but most of them are of no use for sequence work because they cut each molecule randomly. This produces a heterogeneous collection of fragments of varying sizes. What is needed is a way that cleaves the DNA molecule at a few precisely located sites so that a small set of homogeneous fragments are produced. The tools for this are the restriction endonucleases. The rarer the site it recognizes, the smaller the number of pieces produced by a given restriction endonuclease [7, 11].

By using the reverse transcriptase of an enzyme of retroviruses, the produced hybrid RNA-cDNA and the following RNase H digests the partly RNA thread

[11]. The cDNA incurred is deprived of introns and control sequences [1]. The remaining RNA fragments serve as primers for polymerase DNA I, which synthesizes the other cDNA chain [11]. Further proceedings are the same as in the formation of the genomic library; although the clones incurred contain the set of only these genes, which surrendered to the expression in the used cell.

Creating false fragments based on the known amino acid sequence of the protein requires fitting codons that correspond with particular amino acid. For short peptides it will be sufficient to create single strand DNA and hybridize with a second complementary thread. For long peptides, a lot of chemically encapsulated oligonucleotides undergo hybridization; polymerase (a polymerase is an enzyme whose central biological function is the synthesis of polymers of nucleic acids. DNA polymerase and RNA polymerase are used to assemble DNA and RNA molecules, respectively, by generally copying a DNA or RNA template strand using base-pairing interactions) and ligase (ligase is an enzyme that can catalyze the joining of two large molecules by forming a new chemical bond, usually with the accompanying hydrolysis of a small chemical group that is dependent on one of the larger molecules or the enzyme catalyzing the linking together of two compounds) fill the gaps incurred in the helix [24, 42].

Selection of the corresponding gene from the library occurs because of the use of the probe that is complementary to the given fragment of a marked thread of DNA [1]. The clones are transferred to a nitrocellulose or nylon membrane and are brought to denature the DNA through temperature (80°C for nitrocellulose) or UV (for nylon). After adding the marked probe, hybridization occurs with the denatured DNA strands. Unbound probes are then scoured and the sample is dried and subjected to autoradiography. The visible stripe corresponds to the sought gene [8]. The probe can be homologous, which is perfectly complementary to the given DNA fragment, or the probe can be heterologous when the sequence is only very similar to the studied particle [5, 38].

PCR allows for obtaining a lot of chosen fragments of the polynucleotide chain. This sequence remains unknown. Knowledge of the flanking sequences, which are located on both sides of the section, intended for amplification is, however, sufficient. For the reaction mixture, aside from the DNA, large amounts of two types of short oligonucleotides, which are complementary to the flanking sequences and

serve as primers, and heat resistant Taq polymerase are added. Heating the content of the tube to 94°C causes the denaturation of the double helix DNA. Cooling to 50–60°C makes the single strand oligonucleotides bind. After being reheated to 74°C the Taq polymerase begins acting and synthesizes the new chain. Multiple (25–30 times) repeats of these steps causes the creation of millions of copies of the amplified segment [11].

Introduction a gene into vector

A bacterial plasmid can be the vector – spherical autonomous particle DNA, bacteriophage (phage λ , e.g., M13), which is a virus infecting the cells bacterium and cosmid, a plasmid with *cosy* ends [11]. In the case of the long molecules of the DNA of mammals, created artificial chromosome vectors (YACi, Baci, MACS) are created that can fit large fragments [1, 31].

DNA fragments, incurred through endonucleases cuts, do not always have the sticky ends required to connect the two molecules of DNA. They are also missing in the case of cDNA. Subsequently, single strand, complementary homopolymers are formed that are then attached until the ends 3' and 5' of the second chain of the particle with blunt ends with the help of terminal transferase. The other method is to connect linkers, the sequence of double-stranded blunt ends that contain at least one restriction point. After ligation linkers to DNA, they are digested by the appropriate endonuclease, which creates sticky ends [8]. Adapters are used when the cloned DNA particle has the same restriction point as the linkers. Adapters have one blunt end and one cohesive end. End 5' is deprived of the phosphoric group, and the adapters therefore do not connect together in a mixture before connecting to the DNA. After the blunt ends of the adaptors and the fragment of the DNA bind, the polynucleotide kinase joins the phosphoric group to the recalled place and can then reach ligation of the examined DNA from the DNA of the vector [11].

Another example is a vector of the polymer. Polymer-based vectors are biologically safe, have low production costs, and are efficient tools for gene therapy. Although non-degradable polyplexes exhibit high gene expression levels, their application potential is limited due to their inability to be effectively eliminated, which results in cytotoxicity. The development of biodegradable polymers has allowed for high levels of transfection without cytotoxicity [24].

Transfer of recombinant vector into cell

In order to facilitate entering the vector into the bacterial cell, one should make the cell competent. Plunging the cell in CaCl_2 solution or some other salts and then heating for 2 min to 42°C causes it to become more susceptible to accepting the new DNA from the surroundings. Entering the recombined plasmid into the bacterium cell is known as a transformation.

Phage vectors are introduced in the process of transfection or packaging *in vitro*. The transfection also consists of creating competent bacterial cells and heating the solution, but introduces the RF (replicative form) or M13. During *in vitro* packing, it puts up with being in a mixture of recombinant material of λ phages in the protein border. It uses the phage genes that are responsible for the production of capsid proteins and tail.

After obtaining a sufficient amount of proteins required for packaging, complete recombined phages which bacteria are then formed, which attack through the natural way of infection and are entering their DNA sample into them [11].

It is possible to implement the vector in this way by putting it at first in leptosomes, and then by natural fusion of leptosomes with the cell or by precipitation of molecules in the calcium phosphate on the surface of the cell. Fungi, yeast and plants have cell walls that are destroyed with enzymes. Protoplasts are formed that can easily enter the DNA fragments. Treatment of the cell by electroporation, brief electrical pulses, increases the efficiency of the transformation. Leaching of enzymes destroying the cell wall causes the wall to begin to rebuild and the targeted recombinant arises.

Microinjection is a method that enables the new DNA to enter directly into the nucleus of the cell with the micropipette. This applies to animal and plant cells. Biolistics is based on pelting the cell with slices of tungsten or gold associated with the DNA [11].

Selection of transformants and the identification of recombinants

After implementing the vector, bacteria is being cultured to a peculiar base, known as a selective one. The composition depends on what the factor is that allows for the diversity of organisms in which there has been a vector implanted. Often this factor is at least a one-gene plasmid that encodes a protein, excluding the antibiotic. Plating transformants for the fuel containing antibiotics in its composition, which is a sensitive,

vaccinate wild strain. It allows the identification of mutant cells because it will grow despite the earlier presence of harmful substances. Before planting, there is an incubation period at 37°C for an hour from the beginning of the transformation, set in order to create the largest possible number of recombinants.

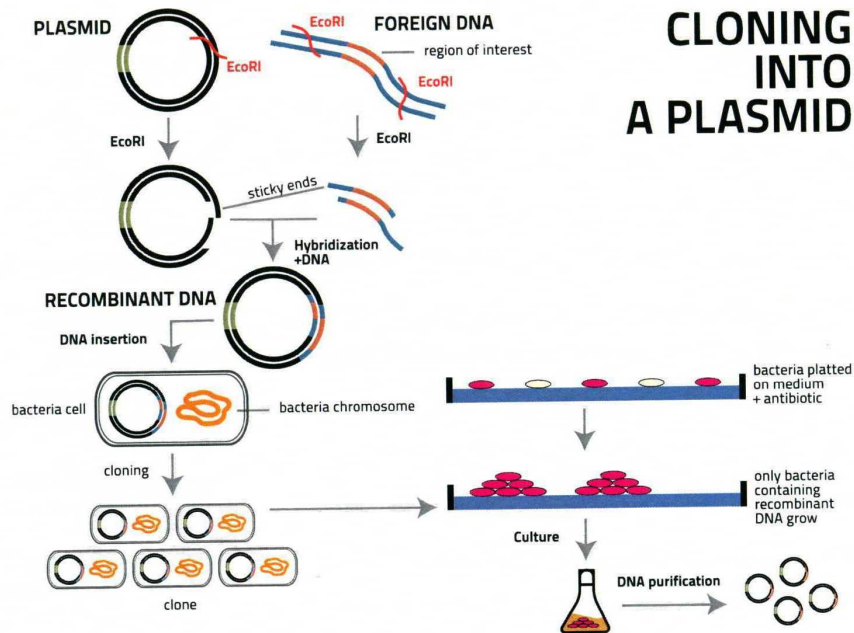
However, not all vectors contain a cloned gene. In order to check which cells are recombinants and have a ligated vector with the gene, insertional inactivation I is used. Cloning of the new DNA fragment can take place in the centre of the naturally present gene in the vector, when it has characteristic restrictive sequences. If the slit gene has encoded resistance proteins to some antibiotic, then cells of the host containing such a vector will not also be immune to it. This happens in the case of plasmid pBR322, which has resistance genes to tetracycline and ampicillin.

The gene of the resistance to tetracycline has a BamHI restriction point, which is then cut. Recombinants containing this vector are therefore insensitive to ampicillin, but sensitive to tetracycline. At first, bacteria are being plated to the base with the ampicillin, where transformants survive. Next, a method of replicas is applied – transformants are transferred to the base with tetracycline. Non-recombined survives only, and we know the position of the recombinants on the previous plate.

Restriction endonuclease *Bam*HI shows a striking resemblance to the structure of endonuclease *Eco*RI, despite the lack of sequence similarity between them. The active site of *Bam*HI is structurally similar to the active sites of *Eco*RI, but the mechanism through which *Bam*HI activates a water molecule for nucleophilic attack may be different [35].

In the case of the pUC8 plasmid resistance genes to the antibiotic aren't being used, although it has a resistance gene to the ampicillin. Present in its genome, the gene *lacZ'* coding part of the β -galactosidase (decomposing lactose and its analog X-gal), is the factor that allows for the determining of the presence of recombinants. Insertional inactivation of *lacZ'* causes the β -galactosidase does not create. Cells are planted at the base with the ampicillin and X-gallim, which decompose by enzyme and cause the growth of the blue products. Accordingly, non-recombined bacteria will form blue colonies, recombinanted – white. This method is called the selection of Lac.

Many λ phages and all M13 also have a *lacZ'* gene. Recombinants can be identified by insertional inactivation as described above. Moreover λ phages are re-

Fig. 1. Steps for recombinant DNA technology

strictive in the gene sequence of cI, and the insertion in this place of a new fragment of DNA causes the colonies grown on the plate will to be clean. On the other hand, when a recombination isn't occurring, the colonies are muddy. λ vectors are determined also as Spi⁺ when they cannot infect the cell which was already infected with the P2 phage. When λ phages are recombined, these become Spi⁻ and are infecting bacteria from P2. Only such cells will now grow on the plate (Fig. 1) [11].

It is also possible to test the initiative of the enzyme directly, of which the gene was itself cloned, if it does not occur naturally in the cell or the use of antibodies to the protein [8] (Tab. 1).

Production of the human insulin

The early success of recombinant DNA technology relies heavily on the elucidation of the biological processes at the molecular level in microbial systems. The first commercial application was realized in the microbial production of human insulin. The primary role of insulin is to control the absorption of glucose from the bloodstream into cells where glucose is utilized as an energy source or converted into glycogen for storage. Insulin's function is to regulate the level of glucose in blood [3, 6, 12, 14].

Insulin is produced and stored in the β cells of the Islets of Langerhans of the pancreas. Insulin is released from storage in the pancreas into the bloodstream [32]. Before the advent of biotechnology, the insulin used for the treatment of type I (insulin-dependent) diabetes mellitus was obtained by extracting the hormone from porcine or bovine pancreatic tissues. In the early eighties, human insulin produced by recombinant technology entered the pharmaceutical market. In one of the approaches the sequences for the A and B chains were synthesized chemically and inserted separately downstream of the δ -galactosidase structural gene controlled by the *X lac* promoter. The construction was such that the insulin chains would be made as fusion proteins joined by a methionine to the end of the P-galactosidase protein. The expression vector also contained an Amp^r marker. The transformants were then screened by plating on a culture medium containing X-gal and ampicillin. The insulin A chain and B chain transformants were grown to harvest the cells in large quantity. The cells were lysed and the insulin A chain and B chain were purified separately. Because the insulin A gene was fused to the δ -galactosidase gene, therefore the insulin protein produced was therefore a δ -galactosidase-insulin hybrid (Fig. 2) [7, 12, 23].

Tab. 1. Examples of drugs produced by recombinant technology

Active molecule	Drug name	Disease
Insulin	Humalog, NovoRapid, Gensulin R, Humulin R, Actrapid HM	for diabetics
Factor VIII	Factor VIII from Bayer	for males suffering from hemophilia A
Factor IX	Factor IX from Bayer	for hemophilia B
Human growth hormone (HGH)	Genotropin, Humatrop, Serostin	for growth hormone deficiency
Erythropoietin (EPO)	Eporex, Epogen	for treating anemia
Interferon	Avonex, Rebif, Betaseron	the hepatitis B virus
α -L-iduronidase (rhIDU; laronidase)	Aldurazyme	mucopolysaccharidosis type I (MPS I; deficiency of α -L-iduronidase) for the treatment of non-neurological symptoms
N-acetylgalactosamine-4-sulfatase (rhASB; galsulfase)	Naglazyme	mucopolysaccharidosis VI (MPS VI 4-sulfatase deficiency of N-acetylgalactosamine, Maroteaux-Lamy syndrome)
Granulocyte colony-stimulating factor (G-CSF)	Neupogen	stimulating neutrophil production (e.g., after chemotherapy) and for mobilizing hematopoietic stem cells from the bone marrow into the blood
Tissue plasminogen activator (TPA)	Activase	for dissolving blood clots
Adenosine deaminase (ADA)		for treating some forms of severe combined immunodeficiency (SCID)
Dornase alfa	Pulmozyme	for cystic fibrosis
Glucocerebrosidase	Ceredase	for type 1 Gaucher's disease
Hepatitis B surface antigen (HBsAg)	Engerix B	to vaccinate against the hepatitis B virus
C1 inhibitor (C1INH)		used to treat hereditary angioedema
Follicle-stimulating hormone (FSH)	Pergonal	to treat fertility issues in women, especially women who are anovular and oligoovular

A more effective method, also using a strain of *E. coli*, involves the production of proinsulin completely whole, instead of separate fragments of insulin synthesis. Proinsulin consists of chains A and B, connected with chain C [16]. The gene encoding proinsulin cloned to *E. coli* cells is also expressed there. The resulting hormone is cleared and then the chain C is proteolytically removed [28, 43].

Insulin Lispro was the first recombinant fast-acting insulin analogue to gain marketing approval. It displays an amino acid sequence identical to native human insulin, with one alteration – an inversion of the natural proline-lysine sequence found at positions 28 and 29 of the insulin B-chain. This simple alteration significantly decreased the propensity of individual insulin molecules to self-associate when stored at therapeutic dose concentrations. The dimerization

constant for Insulin Lispro is 300 times lower than that exhibited by unmodified human insulin. Structurally, this appears to occur as the change in sequence which disrupts the formation of inter-chain hydrophobic interactions critical to self-association. Insulin Lispro was developed by scientists at Eli Lilly (which, along with Novo Nordisk, are the world's largest producers of therapeutic insulin) [22]. The rationale underlying the sequence alteration was rooted in studies, not of insulin, but of the insulin-like growth factor-1. The latter displays a strong structural resemblance to proinsulin, with up to 50% of amino acid residues within the IGF-1 A- and B-domains being identical to those found in comparable positions in the insulin A- and B-chains. When compared to insulin, IGF-1 molecules display a significantly decreased propensity to self-association. Sequencing studies ear-

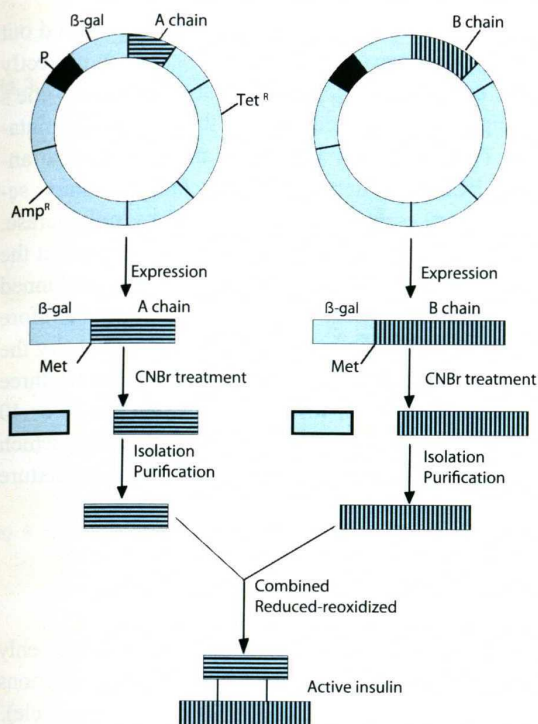


Fig. 2. Production of insulin

lier revealed that the ProlineB28-LysineB29 sequence characteristic of insulin is reversed in IGF-1. It was suggested that, if this sequence difference was responsible for the differences in the propensity for self-association, then inversion of the ProlineB28-LysineB29 sequence in insulin would result in its decreased self-association. Direct experimentation proved this hypothesis accurate. Insulin Lispro is manufactured commercially in a manner quite similar to the 'proinsulin' route used to manufacture native recombinant human insulin. A synthetic gene coding for LysB28-ProB29 proinsulin is expressed in *E. coli*. Following fermentation and recovery, the proinsulin is treated with trypsin and carboxypeptidase B, resulting in the proteolytic excision of the engineered insulin molecule. It is then purified to homogeneity by a number of high-resolution chromatographic steps. The final product formulation also contains *m*-cresol (preservative and stabilizer), zinc oxide (stabilizer), glycerol (tonicity modifier) and a phosphate-based buffer. The commercial product has a shelf life of 2 years when stored at 2–88°C [18, 48].

Production of the medicine against blood clot – ACTIVASE®

Activase® is a drug produced by the Genentech Inc. This pharmaceutical is used in the treatment of myocardial necrosis and pulmonary embolism and is administered to patients within three hours of stroke symptoms. Activase® dissolves clots in the blood vessels.

Activase® is an alteplase that is a tissue plasminogen activator (t-PA) and is produced on a global scale by recombinant DNA technology. The tissue plasminogen activator is glycoprotein, which is composed of 527 amino acids and belongs to a system regulating the blood flow [39, 41]. The t-PA is an enzyme activating the inactive plasminogen to its catalytic form – plasmin. Plasmin is responsible for fibrinolysis, which is a decomposition of thromboses [4, 43]. The production of biotechnological methods uses cDNA corresponding to the natural, human t-PA derived from melanoma cells. cDNA has been introduced into the ovary cells genome of a Chinese hamster (CHO, Chinese hamster ovary), and then carried out in cell culture medium containing gentamicin at a concentration of 100 mg/l. During the breeding, alteplase is excreted into the medium. For leveling the pH a phosphoric acid or a sodium hydroxide is added, while the protein synthesis is carried out by freeze-drying to yield a sterile white powder [19].

Directed mutagenesis

This method allows for making of specific changes in the sequence of the protein recombinant, so that the peptide gains new and beneficial properties. Mutations are most often implemented in the essential areas for the function of the proteins, such as the active centre. Changes can concern even only one amino acid [25]. Accordingly, directed mutagenesis is a part of protein engineering [29, 36].

Mutations can be introduced: insertion, deletion or replacement of one amino acid or the whole significant fragment. There are lots of varieties of mutagenesis, but all of these are based on the fundamental mechanisms.

Mutagenesis using oligonucleotide

This method is used to obtain a point mutation. An interesting gene in the form of a single-stranded oligonucleotide is introduced into the vector – the single-stranded M13 phage [2]. The gene is cloned, then purified and sequenced [11]. Oligonucleotide complementary to the original is created, yet containing a given mutation. This is introduced again into phage M13. The DNA fragment is not perfectly complementary to the vector DNA. Polymerase builds on a complementary chain. The chain creates a double helix and contains the cloned gene, which is a primer for the enzyme at that stage. Phages are introduced into bacteria and their replication occurs [21]. Half of the resulting particles correspond to the original DNA, and the other half contains the mutations. Following the release of phage from the cell, also 50% of them also have a mutated single-stranded molecule. Then, the phages are plated on a plate with bacteria to produce plaques and the original oligonucleotides are used as a probe for hybridization with the mutant gene. The gene can now be transferred to the vector bacteria to produce a modified protein [8, 20, 22, 47].

Synthesis of the artificial gene

If the gene encodes a protein with a short and known sequence we can easily create it in an artificial form. It is then possible to introduce many changes within the sequence, besides the point mutation, as in the above description. This gene is formed in a tube and the mutation can be conducted anywhere. At first, they are coming into existence overlapping oligonucleotides for lengths of 150 bases. Sequences will form appropriately amended gene. These are then folded, and the gaps are filled by DNA polymerase. Ligase creates the missing phosphodiester bonds, forming a complete, double-stranded helix. If the ends of the chain sequences are restrictive, after splitting the corresponding enzyme get sticky ends after it is split and the fragment of the vector is introduced [11, 32].

Polymerase Chain Reaction

Another method, very fast and easy to carry out is the PCR (Fig. 3). As in the case of mutagenesis for using oligonucleotide [17], PCR can make only a single

change in the sequence. Two reactions are carried out in each of the two types of primers; one is perfectly complementary to the template, and the second one's principle is unmatched, so that it is a specific mutation. These particles are mixed and subjected to another PCR reaction, in which complementary sequences are combined and extended by polymerase. The disadvantage of this method, however, is that the Taq polymerase makes errors and new, unplanned mutations can appear. PCR products must therefore be sequenced to determine which molecules carry the correct change [11]. More simply said, there are three major steps in PCR, which are repeated for 30 or 40 cycles. This is done on an automated cyclizer, which can heat and cool the tubes with the reaction mixture in a very short time.

1. Denaturation at 94°C

During the denaturation, the double strand melts openly to single stranded DNA, and all enzymatic reactions stop (for example: the extension from a previous cycle).

2. Annealing at 54°C

The primers are jiggle around, caused by the Brownian motions. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

3. Extension at 72°C

This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, become loose again (because of the higher temperature) and don't provide an extension of the fragment. The bases (complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template) (Fig. 4).

Fig. 3. PCR

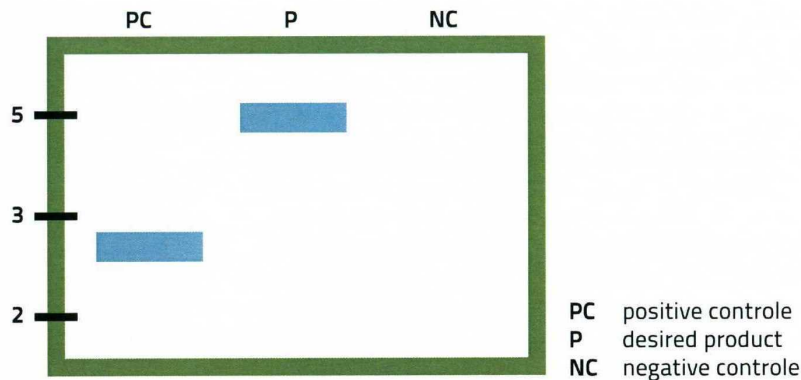
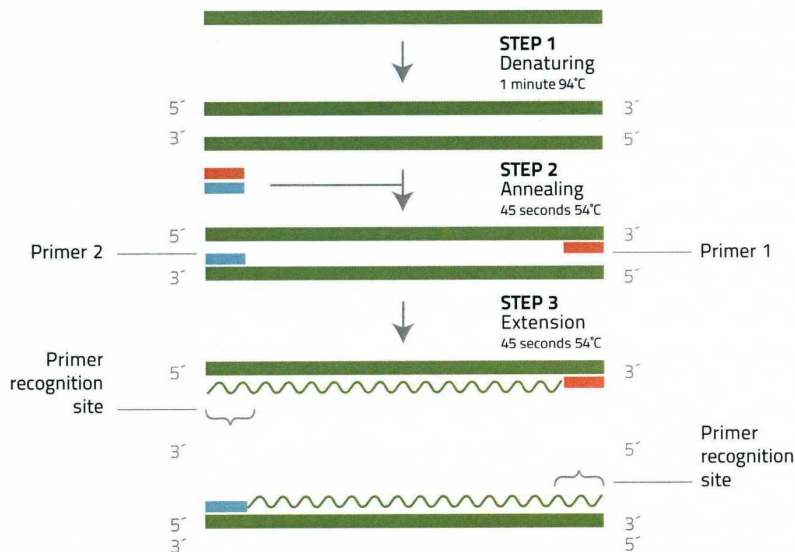


Fig. 4. Steps in PCR



Production of the human somatostatin hormone

Somatostatin inhibits the secretion of somatotropin (growth hormone) by the pituitary gland as well as some other hormones such as insulin and glucagon. It is used to treat acromegaly (excessive production of somatotropin) and its analogs are used as anticancer drugs. It consists of 14 amino acids.

For the production of somatostatin, a method of gene fusion was used, which produced a fusion mRNA. Cloning is conducted with the use of plasmids having the promoter lac and as such, the gene of the somatostatin will undergo to the expression along with β -galactosidase. Since the amino acid se-

quence of the protein is short and well known, it creates an artificial oligonucleotides [42].

It have however made some changes in order to facilitate cloning:

1. For the C-terminal cysteine codon two additional stop codons were included. This ensures that the translation will end after the somatostatin gene.
2. Before the N-terminal methionine there was added alanine codon, which will separate the fragment β -galactosidase from the desired protein and allows their separation by treating the CNBr.
3. The ends of the restriction fragment included sequences of EcoRI and BamHI, which create sticky ends.

Thus, the prepared oligonucleotide is introduced into the vector and is brought to transfection of

E. coli. After incubation, the recombinant fusion protein is selected and purified. CNBr dissects it and yields somatostatin [26, 27, 37].

Production of the ARANESP™ (DARBEPOETIN ALFA)

Darbepoetin alfa is a synthetic form of erythropoietin. It stimulates erythropoiesis (increases red blood cell levels) and is used to treat anemia, commonly associated with chronic renal failure and cancer chemotherapy. Darbepoetin is marketed by Amgen under the trade name Aranesp.

Aranesp (darbepoetin alfa) is an erythropoiesis-stimulating protein that is produced in CHO cells by recombinant DNA technology. Aranesp is a 165-amino acid protein that differs from recombinant human erythropoietin in containing 5 N-linked oligosaccharide chains, whereas recombinant human erythropoietin contains 3 chains. The 2 additional N-glycosylation sites result from amino acid substitutions in the erythropoietin peptide backbone. The approximate molecular weight of darbepoetin alfa is 37,000 D. The finished product is given intravenously or subcutaneously [25].

Conclusion

The presented processes dominate in biotechnological production methods, therapeutics, and recombinant DNA technology as a basic tool for the majority of them, allowing their constant development and improvement. By genomics, the study of the genomes of organisms, and proteomics, plus studying proteins normally synthesized in the cell, produces a lot of information that serves as the basis for the design of new biopharmaceuticals [13, 38, 43, 48]. Drugs produced by biotechnology assume an increasing share among therapeutics. Most biopharmaceuticals are produced using *E. coli*, *S. cerevisiae* and animal cells (as CHO).

Biotechnology is thus a rapidly expanding field, showing the possibility for the effective treatment of diseases that today we may seem incurable.

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