



Regulation of *Pichia pastoris* promoters and its consequences for protein production

Thomas Vogl¹ and Anton Glieder^{1,2}

¹ Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, Graz A-8010, Austria

² Austrian Centre of Industrial Biotechnology (ACIB GmbH), Petersgasse 14, Graz A-8010, Austria

The methylotrophic yeast *Pichia pastoris* is a widely used host for heterologous protein production. Along with favorable properties such as growth to high cell density and high capacities for protein secretion, *P. pastoris* provides a strong, methanol inducible promoter of the alcohol oxidase 1 (*AOX1*) gene. The regulation of this promoter has been extensively studied in recent years by characterizing *cis*-acting sequence elements and *trans*-acting factors, revealing insights into underlying molecular mechanisms. However, new alternative promoters have also been identified and characterized by means of their transcriptional regulation and feasibility for protein production using *P. pastoris*. Besides the often applied GAP promoter, these include a variety of constitutive promoters from housekeeping genes (e.g. *TEF1*, *PGK1*, *TPI1*) and inducible promoters from particular biochemical pathways (e.g. *PHO89*, *THI11*, *AOD*). In addition to these promoter sequence/function based studies, transcriptional regulation has also been investigated by characterizing transcription factors (TFs) and their modes of controlling bioprocess relevant traits. TFs involved in such diverse cellular processes such as the unfolded protein response (UPR) (Hac1p), iron uptake (Fep1p) and oxidative stress response (Yap1p) have been studied. Understanding of these natural transcriptional regulatory networks is a helpful basis for synthetic biology and metabolic engineering approaches that enable the design of tailor-made production strains.

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Corresponding author: Glieder, A. (anton.glieder@acib.at)

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Background

Methylotrophic yeasts like *Pichia pastoris*, *Candida boidinii*, *Hansenula polymorpha* (*Pichia angusta*) and *Pichia methanolica* are widely used host systems for heterologous protein production [1,2]. They unite the benefits of a eukaryotic expression system (efficient secretion and post-translational modifications like glycosylations or the introduction of disulfide bonds) with favorable growth properties of robust unicellular organisms (fast growth on minimal media, no production of intrinsic endotoxins or viral DNAs) [3].

Expression of the respective gene of interest (GOI) for recombinant protein production includes transcription, translation, folding and possible posttranslational modification as well as the correct targeting by the heterologous host organism. The initial transcription of the GOI is often a critical step in heterologous protein production, in both pro- and eukaryotes [4–7]. Therefore, strong and controllable promoters are a crucial tool for efficient heterologous protein production [7,8]. Promoters regulate transcription by providing specific DNA-binding sites for transcription factors (TFs) and the basal transcription machinery (e.g. the RNA polymerase and associated factors). TFs are *trans*-acting factors that can act either as activators or repressors and which bind to *cis*-acting regulatory DNA elements [9]. Even for the more complex eukaryotes, the understanding of the underlying mechanisms has drastically increased over the past decades and light has been shed on both *trans*-acting factors and *cis*-acting sequence elements including their effect on expression and involvement in various transcriptional regulatory circuits [10–12]. This knowledge and random approaches have been used to engineer synthetic promoter libraries. These promoter variants have also been applied in biotechnological protein production as they provide altered regulatory profiles and a range of different promoter strengths, often exceeding wild-type promoters available in a certain host [13,14]. Finding the optimal transcription level by comparing various natural promoters, synthetic variants or tunable promoters is an important aspect to optimize protein production in an expression system. This review is focused on recent findings on promoters and transcriptional regulatory circuits of TFs in the methylotrophic yeast *P. pastoris* and their application to improve heterologous protein production.

One of the most favorable properties of *P. pastoris* and the other methylotrophic yeasts are the strong methanol inducible promoters present in these organisms. Certain genes of the methanol utilization (MUT) pathway are completely repressed under growth on repressing carbon sources (e.g. glucose), but are strongly

induced on methanol, constituting up to 20–30% of total intracellular protein [15,16]. This tight regulation is exerted on the transcriptional level, making MUT promoters ideal tools for heterologous protein production [17]. Therefore also most of the published knowledge about the regulation of *P. pastoris* promoters is focused on the most commonly used classic *P. pastoris* promoters, the promoters of the MUT pathway gene *alcohol oxidase 1* (P_{AOX1}), its rarely used homolog P_{AOX2} , and also the glycolysis gene *glyceraldehyde 3-phosphate dehydrogenase* (P_{GAP}), which was described as a constitutive promoter in the early days of development. However, although less characterized, alternative promoters offer advantages for some proteins and also first regulatory networks for important folding processes and stress response in *P. pastoris* have been reported.

Classic *P. pastoris* promoters

AOX1 promoter

Physiological role and regulation of AOX1 expression

Alcohol oxidase (Aox) catalyzes the first step in the metabolism of methanol, the conversion of methanol to formaldehyde. This reaction is taking place in the peroxisomes and involves the incorporation of oxygen and release of hydrogen peroxide as a side product [3]. The oligomeric flavoenzyme alcohol oxidase has however a low affinity for its substrates methanol and oxygen [18,19]. Therefore large amounts of the enzyme are needed to sustain growth on methanol and AOX accounts under these conditions for about 5% of total mRNA and 30% of total soluble protein. Cells grown on repressing carbon sources (e.g. glucose, glycerol, ethanol) [20] show however a complete lack of AOX activity. This tight regulation is exerted on the transcriptional level, as AOX mRNA was described as undetectable in cells grown on repressing carbon sources [21–23].

P. pastoris has two alcohol oxidase genes, *AOX1* and *AOX2*, of which *AOX1* is much more strongly transcribed than *AOX2* [22,23]. As P_{AOX1} is a remarkably strong promoter and at the same time directly controllable by simply changing the inexpensive carbon source [24], it is the most commonly used promoter for heterologous protein expression in *P. pastoris*. The highest expression levels of heterologous proteins in *P. pastoris* have been achieved with P_{AOX1} (up to 22 g/l intracellular and 15 g/l secreted protein) [25,26]. Concerning controlled bioreactor cultivation, the tight regulation of P_{AOX1} is used to enable cell growth to high densities on a repressing carbon source without heterologous protein expression hampering the cellular metabolism.

On the basis of the observation that P_{AOX1} induction requires the complete lack of a repressing carbon source and the presence of the inducer methanol, the current regulatory model involves three regulatory states. Catabolite repression suppresses expression as long as a repressing carbon source is present. Even if methanol is fed, the promoter is still repressed. As soon as the repressing carbon source is depleted or at low concentration in fed-batch processes, the promoter reaches the state of derepression. In *P. pastoris*, under these conditions the native *AOX1* promoter is still only slightly derepressed (2–4% of induced levels) and only methanol addition leads to full induction, which is the third state of regulation [20,27,28]. Fig. 1 shows a schematic representation of P_{AOX1} . The most commonly used sequence from the *Bgl*II restriction site downstream and features like the TATA box and the transcription start site are highlighted. Tschopp *et al.* [27] and Stroman *et al.* [29] had initially used a 1.1 kbp *Pst*I/*Bam*HI P_{AOX1} fragment (the respective sites are shown in Fig. 1).

Despite the favorable properties of P_{AOX1} and the basic understanding of its regulatory modes, only in recent years light has been shed on the molecular basis of P_{AOX1} transcriptional regulation, thereby enabling new tools and concepts for *P. pastoris* based gene expression. A series of *cis*-acting DNA sequences and also the first two transcriptional activators Mxr1p and Prm1p were identified. Furthermore there was also evidence for regulatory roles of a zeta crystalline and hexose transporters in P_{AOX1} regulation.

Cis-acting regulatory sequence elements

Cis-acting regulatory elements in the promoters of methanol regulated genes have been studied in different methylotrophic

yeasts such as *H. polymorpha* [30,31], *C. boidinii* [32,33] and also extensively in *P. pastoris*. Research in *P. pastoris* has mainly focused on P_{AOX1} and P_{AOX2} by performing deletion and insertion studies or comparative sequence analysis. In addition an early study was performed using a previously uncharacterized *P. pastoris* strain [34]. An alcohol oxidase gene, named *ZZA1*, was identified by hybridization with an *AOX1* oligonucleotide and the promoter of *ZZA1* showed 66% identity to P_{AOX1} . This promoter was used to express an α -amylase gene in *S. cerevisiae* to produce ethanol from starch. The authors identified putative transcription factor binding sites (TFBS) in the P_{ZZA1} sequence and also highly conserved sequence motifs between P_{ZZA1} and the promoters of methanol inducible genes from various organisms such as *P. pastoris* *AOX1*, *AOX2*, *H. polymorpha* *MOX*, *DAS* and *C. boidinii* *S2 AOD1*. It was argued that these conserved motifs resemble the TFBS of *S. cerevisiae* Gcr1 and Rap1, both activating factors. Notable unique elements from P_{ZZA1} include a putative upstream repressing site and a putative binding site for a *S. cerevisiae* TF encoded by the *ADR1* (alcohol dehydrogenase regulator 1) gene. Adr1p activates in *S. cerevisiae* various glucose repressed genes such as alcohol dehydrogenase (*ADH2*) [35], fatty acid metabolism and peroxisomal biogenesis genes [36,37]. This indicates that the *P. pastoris* homolog of ScAdr1p could have adapted to the regulation of methanol induced genes. Lin-Cereghino *et al.* identified later indeed a positive regulator of methanol inducible genes, Mxr1p (methanol expression regulator 1), which is a homolog of ScAdr1p (see also section 'Trans-acting factors') [28].

In conclusion, the P_{ZZA1} study indicated first possible regulatory sequences which were however only identified by sequence

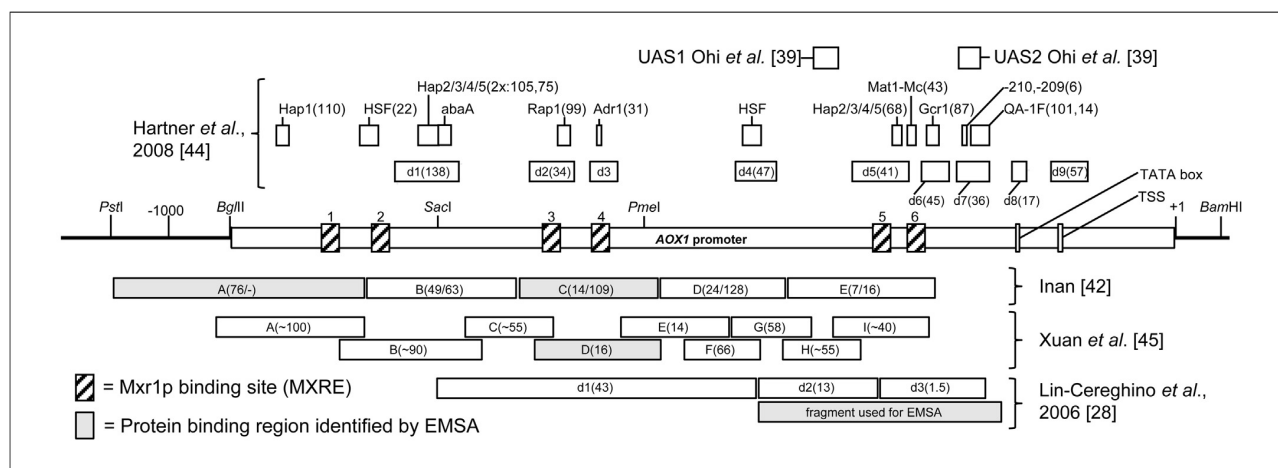


FIGURE 1

Cis-acting sequence elements of the *AOX1* promoter and their effect on expression. A schematic representation of the most commonly used *AOX1* promoter sequence (from *Bgl*II restriction site to start codon) is given. The *Pst*I, *Bgl*II, *Sac*I, *Pme*I, *Bam*HI restriction sites, TATA box and transcriptional start site (TSS) are shown. The translational start is indicated by +1. Sequence elements identified by systematic deletion studies by Inan [42], Xuan *et al.* [45] and Lin-Cereghino *et al.* [28] are shown below the strand ruler. Putative TFBS identified by Hartner *et al.* [44] using MatInspector software and related deletion studies are shown above the strand ruler. If reported, the effect of the deletions is given in parentheses in percent of wild-type promoter activity (under methanol induction). Inan performed sequential and drop out deletions, and both effects are given separated by a slash. Hartner *et al.* tested in total 46 deletion variants; here only deletions of the putative TFBS and longer stretches (d1 to d9) are shown. Hartner *et al.* did not provide percent of wild type activity values for all deletions, therefore the values shown here were calculated from Supplementary Table 3 of Hartner *et al.* (using the values at 72 hours induction). There are three putative Hap2/3/4/5 binding sites present, the first two are close together, and therefore just shown as one site. In case of the putative QA-1F site, the first value represents the deletion only of the putative TFBS, and the second value represents a deletion of additional six bp adjacent to the TFBS. Also the putative UAS suggested by Ohi *et al.* are depicted [39]. Elements shown in gray are bound by proteins, as they caused a band shift with cell extracts from cultures grown on methanol in the respective studies. Experimentally verified Mxr1p binding sites (MXRE, MXR response elements) are shown as dashed boxes and numbered according to Kranthi *et al.* [48].

comparison, but not verified by deletion or insertion studies [34]. Practically all other *P. pastoris* related research was performed using strains based on GS115/X33/KM71, CBS7435/NRRL Y-11430 or DSMZ 70382 and not the unique isolate bearing P_{ZZA1} . Furthermore P_{ZZA1} characterization was only described for the host *S. cerevisiae* [38], but not *P. pastoris* and due to the low sequence identity of 66% it is difficult to transfer the P_{ZZA1} derived results directly to P_{AOX1} .

The first deletion study in *P. pastoris* was performed by Ohi *et al.* using the promoter of the $AOX2$ gene [39]. In early studies, Aox1p accounted under all tested growth conditions for most of the methanol oxidizing activity and no clear physiological role of Aox2p was found. Deletion of $AOX1$ resulted in a strongly decreased growth rate on methanol, whereas the deletion of $AOX2$ retained a wild type like growth. The two genes have 97% identity on the amino acid level but no obvious similarities in their promoter regions. It was furthermore shown that the difference in Aox activities is the effect of transcriptional regulation. This was done by placing the coding sequence (CDS) of $AOX2$ under control of P_{AOX1} . The respective strain showed the same growth rate as the wild type, whereas the reverse exchange of the $AOX1$ CDS under control of P_{AOX2} caused a strong growth defect [23].

Despite their different sequence and dissimilar expression levels, P_{AOX1} and P_{AOX2} show the same basic regulation pattern, as both require the lack of a repressing carbon source and presence of methanol for induction [23,39]. Therefore P_{AOX2} based studies also appear relevant for understanding the regulation of P_{AOX1} . Ohi *et al.* identified negative and positive regulatory elements of P_{AOX2} by deletion and addition of sequence stretches [39]. These studies indicated one upstream activating sequence (UAS) and two upstream repressing sequences (URS). The identified UAS showed similarities with P_{AOX1} and also the promoters from MUT genes of other methylotrophic yeasts such as *C. boidinii* $AOD1$ and *H. polymorpha* DAS . Although it might be easier to improve a weak promoter like P_{AOX2} than the exceptionally strong P_{AOX1} promoter [17], remarkable progress was made in the understanding of methanol inducible promoters. Shortened promoter variants with added UAS showed a more than 100-fold increase in human serum albumin (HSA) expression (from 1 to 120 $\mu\text{g/ml}$). Whether such an improvement is transferable to the strong P_{AOX1} , where several mg/ml HSA can be obtained [40,41], remained unclear.

The first P_{AOX1} based systematic deletion studies were performed in frame of a PhD thesis [42] and a related patent [43]. Inan [42] truncated the P_{AOX1} sequence from the 5' end by sequentially deleting five fragments named A to E (Fig. 1). The stepwise decrease of the promoter length concomitantly reduced the methanol induced expression of the reporter lacZ. This classical truncation approach for promoter studies resulted in generally lower lacZ activities, because large regions, probably containing several TFBS, were deleted in each individual truncation variant. Therefore internal 'dropout' deletions of fragments B to E were also performed, leaving the 5' promoter part upstream of the deleted fragment intact. Deletions of fragments B and E resulted in decreased reporter activity (63 and 16% of wild-type P_{AOX1} activity were observed), whereas deletions of fragments C and D increased the activity to 109 and 128%. This seems to contradict results of the deletion of the d1 region by Hartner *et al.* [44]. The d1 region is

part of region B deleted by Inan but a d1 deletion increased the promoter activity significantly. This indicates the location of several important binding sites in region B and a combined effect of lost positive and negative sites upon deletion of this region. All fragments were tested for protein–DNA interactions with cell extracts from methanol induced and ethanol repressed cultivations. In these electrophoretic mobility shift assay (EMSA) experiments only fragments A and C showed specific DNA–protein complexes, with cell extracts from methanol induction. These interactions were considered to arise from interactions with different proteins, as fragment A did not compete binding of fragment C and vice versa. Unfortunately, the bound proteins were not further characterized [42]. Three fairly long fragments of P_{AOX1} were sequentially deleted by Lin-Cereghino *et al.* [28] (Fig. 1) to identify binding regions of the TF Mxr1p (discussed in the section about *trans*-acting factors). However, while this study mapped a putative Mxr1p binding site, the deletion studies of Inan [42] and Xuan *et al.* [45] achieved a higher resolution by deleting shorter sequence stretches and also included internal drop out deletions. This allowed to map also none additive effects, which are solely obtained by sequential deletion studies.

An alternative design for deletion studies was followed by Hartner *et al.* [44,46]. In contrast to sequential deletions of large sequence stretches, putative regulatory *cis*-acting elements of the P_{AOX1} sequence were identified by sequence similarities to known TFBS from other organisms. Subsequently systematic single- and multisite deletions of those short sequence stretches were performed (Fig. 1). Thereby a first promoter library was created, that included 46 variants showing between ~6 and >160% of the wild-type promoter activity employing GFP as a reporter. For the most important promoter variants single copy integration of the expression cassette was verified by qPCR and the results were reevaluated with other reporter proteins. Transcript levels have not been determined but because GFP expression usually increases with copy numbers it might be speculated that strong GFP fluorescence also indicates high mRNA levels. In the same library variants with different regulatory patterns (e.g. derepression) were also identified. The deletions of varying short stretches (2–59 bp) of these TFBS and longer internal fragments (termed d1 to d9) showed different effects. For several putative TFBS (e.g. HSF, Adr1, Mat1-Mc), a clear reduction of P_{AOX1} driven expression was observed (22–43% fluorescence intensity compared to wild-type promoter driven GFP expression, Fig. 1). In one case the deletion of only two bp (at position –209 and –210) abolished GFP expression almost completely (only 6% of wild type fluorescence left) indicating the high importance of this putative TF binding site.

However, improvements of the strong $AOX1$ promoter could also be achieved, for example, by deletion of a longer stretch at the 5' end (fragment d1). Combination of multiple beneficial deletions also showed additive effects, and the best variant reached more than 160% of the wild-type promoter activity. The authors reasoned that deleted stretches leading to decreased expression might be binding sites for activators. Increased GFP fluorescence by contrast indicated possible repressor binding sites. Therefore they designed synthetic promoters, by fusing various putative TFBS of activators to a basal core promoter region. Compared to the core promoter alone, several synthetic promoters showed increased expression levels and altered regulatory patterns. Namely a tighter

glucose dependent repression, and enhanced expression under both derepressing and induced conditions have been achieved. Because all variants were made by deletions, structural effects in the DNA or disturbance of the interaction of different *trans*-acting proteins cannot be excluded.

Interestingly, the deletion variants d6 and d6* also showed a notable altered regulation. They caused increased expression after the carbon source was depleted (derepression). This variant still required methanol for full induction, but the protein production started earlier during the production process resulting for example in twice as much active horseradish peroxidase as the control employing the wild-type promoter [44].

On the basis of the findings of Hartner *et al.* [44], a similar strategy of fusing *cis*-acting elements to a core promoter was also applied by Ruth *et al.* and led to higher productivity and yields of porcine trypsinogen using P_{AOX1} derived variants [47]. Previously characterized sequence elements from Hartner *et al.* and novel regulatory elements were fused to the P_{AOX1} core promoter sequence. These new variants showed up to 218% activity of the wild-type P_{AOX1} and also altered regulation patterns. Most notably a derepressed variant reached without methanol induction 77% of the wild-type activity after 96 hours of methanol induction. However, it is notable that there were multiple copies of this variant present, but only one copy of the wild-type P_{AOX1} was present. Nevertheless this was the first proof that very short semisynthetic promoters built on the basis of a natural core promoter can be used to obtain higher titers of some target proteins. In addition this variant was also shown to be applicable for a methanol-independent fed-batch process.

Various regulatory sequences affecting P_{AOX1} expression were also identified by Xuan *et al.* They deleted nine adjacent fragments, named regions A to I (Fig. 1) [45]. In contrast to the studies of Inan and Lin-Cereghino *et al.*, these fragments were overlapping [28,42]. Most deletions led to a decrease of GFP reporter activity and decreased transcript levels as determined by RT-qPCR. Notably the deletion of regions D and E led to only 16% and 14% activity of the wild-type promoter under methanol induction. All fragments were analyzed for DNA–protein interactions by performing an EMSA. Cell extracts prepared from cultures grown on methanol or glucose and methanol displayed a specific band shift only with region D, but none of the other tested sequence elements. The authors noticed that a stretch of region D showed similarities to the TFBS of the transcriptional activator Adr1p from *S. cerevisiae* (also Hartner *et al.* had identified a putative Adr1p binding site in this region [Fig. 1] [44]). Additionally, insertion of three adjacent copies of region D to the region D deletion variant increased the reporter protein level to 157% of the wild-type promoter. These results indicated that region D is the binding site for a transcriptional activator involved in methanol induction. Lin-Cereghino *et al.* had shown that Mxr1p, the *P. pastoris* homolog of Adr1p, is a key activator of methanol induced genes [28]. Therefore Xuan *et al.* argued that the TFBS of Adr1p and Mxr1p could also be conserved and region D might be a binding region for Mxr1p. Indeed Kranthi *et al.* [48] showed independently by EMSA and DNase I footprinting that region D contains even two Mxr1p binding sites (discussed in detail in the following section *trans*-acting factors, see also Fig. 1).

In summary, these sequence-based approaches identified *cis*-acting regulatory elements, improved the already strong P_{AOX1} and

also provided new variants showing different regulatory patterns. Additionally, more simple short promoters composed of a basic core promoter sequence and additional positive regulatory sequences have been obtained. Generally, the results of the different studies match fairly well, but there are also a few notable discrepancies. Inan [42] and Xuan *et al.* [45] deleted approximately the same fragment (InanC and XuanD, Fig. 1). However, the dropout deletion of XuanD decreased the activity to 16% of the wild-type promoter, whereas the dropout deletion of InanC retained wild type like activity (109%). Consequences to the DNA structure influencing the interaction of DNA-binding proteins might be one possible explanation. Deletions by Hartner *et al.* [44] in this region also caused a loss of activity (e.g. fragment d2 only 34% of wild type activity). Notably, for both the InanC and XuanD fragment DNA–protein interactions were demonstrated in EMSA experiments with cell extracts from methanol grown cultures. Kranthi *et al.* [48] later identified two Mxr1p binding sites (MXRE3 and MXRE4, MXR response element) in the region covered by InanC and XuanD using the recombinantly produced DNA-binding domain of Mxr1p (Fig. 1 and also the next section about *trans*-acting factors). This suggests that the bandshifts arise from Mxr1p binding and that this region is crucial for methanol-dependent P_{AOX1} induction. Concerning the other Mxr1p binding sites MXRE1, 2, 5 and 6, the results from the EMSAs were somewhat inconsistent. Fragment A of Inan caused a band shift with methanol grown cells, but the experiments of Xuan *et al.* with fragment A did not confirm these results. InanA is longer than XuanA, which might explain the difference. However, MXRE1 identified by Kranthi *et al.* [48] lies within both regions (Fig. 1). As Inan showed that the binding events to region A and C are not competing each other, and InanC is most probably bound by Mxr1p, this would suggest that a different protein is binding to InanA, possibly in a region upstream of the *Bgl*III site which was not studied by others. Lin-Cereghino *et al.* [28] identified a Mxr1p binding fragment close to the 3' end of the promoter, using a strain overexpressing Mxr1p. Fragment E of Inan also lies in this region, but did not show a band shift, possibly because Mxr1p was not overexpressed in this study. Kranthi *et al.* [48] identified two Mxr1p binding sites (MXRE5 and 6) lying exactly in InanE and the fragment used by Lin-Cereghino *et al.* [28]. Taking these results together suggest that Mxr1p binds MXRE3 and 4 with higher affinity than MXRE1, 2, 5 and 6, because fragments containing MXRE3 and 4 formed a band shift directly with cell extracts of wild type strains, whereas a fragment containing MXRE5 and 6 could only cause a bandshift upon overexpression of Mxr1p. Determination of the Mxr1p dissociation constant (K_d) by Kranthi *et al.* showed indeed that MXRE4 has a lower K_d than MXRE6 (70 vs. 150 nM) [48]. MXRE1 even had a higher affinity than MXRE4 and 6 (K_d of 50 nM). It is also striking that the Mxr1p binding sites seem to be organized in tandem (Fig. 1). MXRE1&2, 3&4 and 5&6 are arranged in close proximity, however the significance (e.g. for cooperative binding) has not been investigated.

Concerning the outcome of the deletion studies, it is interesting to see, that the two different sequence-based approaches applied by Inan [42] and Xuan *et al.* [45] versus Hartner *et al.* [44] displayed similar results. Hartner *et al.* [44] based their analysis on literature research and computational analysis relying only on deletions of putative TFBS, whereas Inan and Xuan *et al.*

performed a systematic deletion analysis by dividing P_{AOX1} into adjacent overlapping fragments (Fig. 1). Still the best variants of both studies reached similar expression levels compared to the wild-type (Hartner *et al.* [44] >160%, Xuan *et al.* [45] 157%). Nevertheless the deletion of short predicted TFBS delivered different results for specific regions than deletions of larger regions, which resulted only in combined effects of several TFBS. In addition the knowledge of specific short positive and negative regulatory sequences as well as the known Mxr1p binding sites allow the design of short synthetic promoters.

All these studies aimed to identify UAS and URS relevant for transcriptional regulation, thereby focusing on sequences upstream the transcription start site and the TATA box core promoter region. Notably, the 5' untranslated region (5' UTR), which effects translation initiation and mRNA stability, was left mostly untouched (Fig. 1). Recently, Staley *et al.* investigated the effect of the *AOX1* 5' UTR on expression by performing deletion and addition studies in a similar fashion as the promoter based studies [49]. This revealed that the *AOX1* 5' UTR affects mostly translational efficiency, but not mRNA stability as determined by Northern blotting and that there are both positive and negative regulatory elements present.

Trans-acting regulatory factors

The sequence-based approaches of identifying *cis*-acting sequence elements, have to some extent given indications about *trans*-acting DNA-binding proteins and possibly involved TFs. Inan [42] and Xuan *et al.* [45] showed that certain sequence stretches of P_{AOX1} form a DNA-protein complex with cell extracts from methanol induced cells. However, the proteins involved had not been purified or characterized by mass spectroscopy, leaving their identity unknown.

The first TF involved in P_{AOX1} regulation in *P. pastoris* was identified by Lin-Cereghino *et al.* and was named methanol expression regulator 1 (Mxr1p) [28]. *MXR1* was shown to be crucial for the activation of numerous genes involved in MUT and peroxisome biogenesis (*PEX* genes). Initially a *mxr1* mutant was obtained by random mutagenesis in a search for strains defective in peroxisome biogenesis [50]. In contrast to other *pex* mutants, the *mxr1* mutant could not induce P_{AOX1} . Deletion of *MXR1* in the wild type strain resulted in abolished growth on the peroxisomal substrates methanol and oleate. In conclusion, the *mxr1* deletion strain showed under methanol induction highly reduced protein and mRNA levels of *AOX1* and other genes involved in MUT, indicating that Mxr1p is a key regulator of methanol induced genes [28].

Mxr1p was found to show similarities to the zinc finger DNA-binding domain and other motifs of *S. cerevisiae* Adr1p, a TF involved in regulation of glucose repressed genes needed for growth on nonfermentable carbon sources [35–37]. Notably, already in the first P_{AOX1} related sequence based deletion study using P_{ZZA1} [34], the authors had identified putative Adr1p binding sites in this *P. pastoris* alcohol oxidase promoter and discussed its significance (see previous section about *cis*-acting sequences). Also later sequence-based studies of P_{AOX1} identified putative Adr1p binding sites [44,45]. Lin-Cereghino *et al.* reasoned that *P. pastoris* Mxr1p is a homolog of *S. cerevisiae* Adr1p and has evolutionary adopted to regulate genes involved in the methanol metabolism [28].

The binding of Mxr1p to P_{AOX1} was first evaluated by Lin-Cereghino *et al.* by investigating a putative regulatory region identified by deletion studies [28]. An EMSA experiment showed that cell extracts of an Mxr1p overexpressing strain grown on methanol caused a band shift with a 243 bp long region of P_{AOX1} (Fig. 1). However, as only three fairly long fragments (d1 to d3) of P_{AOX1} were tested by sequential deletion studies, the presence of multiple binding sites could not be ruled out.

Indeed, a systematic analysis for Mxr1p binding sites in P_{AOX1} by Kranthi *et al.* [48], revealed six binding sites (termed MXRE, Mxr1 response elements) by EMSA and DNaseI footprinting experiments (Fig. 1) [48]. These studies were performed *in vitro* using a 150 AA N-terminal fragment of Mxr1p, which contained the zinc finger DNA-binding domain and was produced recombinantly in *Escherichia coli*. The P_{AOX1} sequence was divided into 16 fragments, six of which showed a band shift with Mxr1p. The minimal Mxr1p binding sites in these six fragments were identified by DNaseI digestion and found to be approximately 20 bp long containing a 4 base pair 5' CYCC/GGRG 3' core element.

These results match previous research as the Mxr1p binding fragment identified earlier by Lin-Cereghino *et al.* [28] was shown to even contain two Mxr1p binding sites. Also the putative TFBS of ScAdr1p identified by Hartner *et al.* [44] was confirmed to be a Mxr1p binding site.

In a following study Mxr1p binding sites were also identified in the promoters of *dihydroxyacetone synthase* (*DAS*) and *peroxin 8* (*PEX8*) [51]. As these two genes are involved in the MUT pathway and peroxisomal biogenesis, the results emphasize the key regulatory function of *MXR1* proposed earlier [28].

MXR1 seemed to be regulated post-translationally by the subcellular localization, as it was constitutively transcribed at low levels and found to localize to the cytosol in cells grown on glucose but to migrate to the nucleus upon shift to methanol or oleic acid. However, also when cells were grown on glycerol or ethanol Mxr1p was localized in the nucleus, while *AOX1* expression was still repressed on these carbon sources. Therefore regulation of *MXR1* cannot solely rely on the subcellular localization of the Mxr1p [28].

Very recently Parua *et al.* have shown that a *P. pastoris* 14-3-3 protein (GenBank accession no. CCA38880) is involved in the regulation of Mxr1p in a carbon source dependent way [52]. 14-3-3 proteins are highly conserved in all eukaryotes and regulate signaling proteins involved in diverse cellular processes. Typically 14-3-3 interaction depends on the phosphorylation of serine or threonine residues of the target protein. The name '14-3-3' dates back to the initial identification in frame of the systematic classification of brain proteins and refers to the purification fraction number in which these proteins were found [53,54]. 14-3-3 proteins have also been studied in yeast [55,56] and are notably involved in the regulation of *S. cerevisiae* Adr1p, the homolog of *P. pastoris* Mxr1p [57]. The *S. cerevisiae* 14-3-3 proteins Bmh1 and Bmh2 bind to the regulatory domain of phosphorylated Adr1p, thereby inhibiting it. A similar role was found for a 14-3-3 protein in Mxr1p regulation in *P. pastoris* [52]. A sequence comparison showed that Mxr1p contains a conserved 14-3-3 binding motif, that is highly similar to the respective region of Adr1p. An interaction between Mxr1p and the 14-3-3 protein was shown by pull down assays and found to depend on the phosphorylation of a

conserved serine residue of Mxr1p. 14-3-3 binding to Mxr1p did not affect DNA binding of Mxr1p, indicating a post-DNA binding regulatory mechanism (e.g. possibly affecting the recruitment of RNA polymerase II). Carbon source dependent binding of the 14-3-3 protein to Mxr1p seemed to inhibit the expression of Mxr1p regulated genes. A disruption of the interaction between 14-3-3 with Mxr1p by mutating the crucial serine residue of Mxr1p to alanine, led to an elevated expression of Mxr1p regulated genes on repressing carbon sources. The activating effect was clearly stronger on ethanol than on glucose and glycerol, which indicated that 14-3-3 inhibits Mxr1p regulated gene repression especially in the presence of ethanol [52]. This is an interesting finding, as glucose and ethanol were already considered before to repress MUT genes via different mechanisms in methylotrophic yeasts [58,59]. Therefore it seems probable that there exist additional regulatory factors, depending on the carbon source. Possible differences in transcript levels of 14-3-3 in methanol grown cells compared to cells grown on glucose or ethanol have not been discussed. Consequently, elucidating the exact physiological role of 14-3-3 and more notable also the factors involved in repression under other carbon sources than ethanol will require further studies.

Besides Mxr1p, another *trans*-acting factor was revealed in a patent by Takagi *et al.* [60]. Deletion of a gene referred to as *positive regulator of methanol* (*PRM1*, annotated in the *P. pastoris* CBS7435 genome sequence as uncharacterized transcriptional regulatory protein YIL130W, GenBank accession no. CCA40959.1) resulted in a growth defect on methanol but not glucose. This deletion also reduced P_{AOX1} and P_{DAS} (the *DAS* promoter is described in more detail in the section 'Alternative promoters') expression levels under methanol induction (~50-fold decrease of the activity levels of a phytase reporter gene for P_{DAS}). These findings indicated an activating role of Prm1p in regulation of MUT genes. Interestingly, constitutive expression of Prm1p and when grown on glucose resulted in an increase in activity of a phytase gene under control of P_{AOX1} or P_{DAS} . Thus a methanol-free expression from methanol inducible and natural glucose-repressed promoters was achieved by simply expressing the putative transcriptional activator Prm1p. In detail, the P_{AOX1} driven expression of the phytase was achieved by constitutively expressing *PRM1* under control of P_{GAP} and growth on glucose containing medium. A 1.86- to 3-fold increase of phytase gene expression compared to a not *PRM1* overexpressing strain was observed in individual transformants. However, the expression level was not compared to a wildtype strain expressing the reporter under control of P_{AOX1} under methanol induction, leaving the significance of these results unclear. In addition the growth behavior of strains constitutively overexpressing this TF was not described.

The corresponding experiment using P_{DAS} to control the phytase expression, led in different clones to a 7.6- to 44-fold increase of reporter gene expression compared to a not *PRM1* overexpressing strain. The expression compared to methanol induction was again not investigated.

The authors of this patent application did not provide experimental evidence for the mode of action, but argue that Prm1p is binding to the promoters of methanol inducible genes, so the role of Prm1p as a TF is not fully verified.

Additionally to the *PRM1* coexpression experiments, constitutive expression of *MXR1* under control of P_{GAP} was also investigated

under growth on glucose. This resulted in a 7.6-fold increased activity of the phytase expressed from P_{AOX1} compared to the non *MXR1* coexpressing strain. Again no comparison to methanol induction or comparative biomass production was performed. The 7.6-fold increase might still account only for a low absolute expression level, as P_{AOX1} is increased ~1000-fold from glucose to methanol [27]. Furthermore, the results of the *MXR1* coexpression seem to oppose previous findings by Lin-Cereghino *et al.*, where overexpression of *MXR1* under control of P_{AOX1} caused a growth defect on methanol and inhibited expression of MUT genes [28]. Additionally, Mxr1p was shown to localize to the cytoplasm when grown on glucose and to migrate only upon shift to gluconeogenic carbon sources to the nucleus. This raises the question how constitutively overexpressed Mxr1p reaches the nucleus during growth on glucose. Therefore the findings by Takagi *et al.* and Lin-Cereghino *et al.* [28] are not entirely consistent and would require further investigation to clarify the activating mechanism of Mxr1p.

In addition to the activators Mxr1p and Prm1p, a hexose transporter responsible for glucose and fructose uptake has also been shown to be involved in P_{AOX1} regulation [61]. Two hexose transporter homologs, *HXT1* and *HXT2*, were identified in *P. pastoris* and shown to complement a *S. cerevisiae* mutant deficient in monosaccharide uptake. A *P. pastoris* *hxt1* deletion strain showed P_{AOX1} expression when grown on glucose, therefore indicating abolished catabolite repression. This deletion strain showed still a wild type like growth behavior on glucose, indicating that the derepression was not simply caused by lower intracellular glucose levels. Similar regulatory roles of hexose transporters have been studied extensively in other yeasts such as *S. cerevisiae* [62] and notably also the methylotrophic yeast *H. polymorpha* [63,64]. Deletion of the *H. polymorpha* hexose transporter homolog HpGCR1 led to the constitutive expression of the *H. polymorpha* alcohol oxidase (*HpAO*) gene and other peroxisomal proteins when grown on glucose [63]. Also a *H. polymorpha* *hxt1* deletion strain showed weak transient derepression of *HpAO* [64].

Another *trans*-acting factor possibly involved in P_{AOX1} regulation is zeta crystalline (Zta1), a single-stranded DNA (ssDNA)-binding protein [65]. The authors were looking for proteins binding to the P_{AOX1} sequence by EMSA and thereby identified a protein binding to the region of -288 to -115 of P_{AOX1} . Subsequent sequencing of a tryptic fragment identified the protein as a homolog of *E. coli*, *S. cerevisiae* and mammalian Zta1p. Zta1p from cell extracts or recombinant production showed an identical binding behavior to P_{AOX1} ssDNA. Interestingly, Zta1p binding is abolished in presence of NADPH but not NADH *in vitro*. As Zta1p is a NADPH-dependent quinone oxidoreductase and the homologs in other organisms are upregulated under oxidative stress [66], it could play a similar role in P_{AOX1} regulation [65]. Remarkably, cytochrome c, another redox protein and component of the mitochondrial electron transport chain, was shown to bind sequence specific to the *AOX2* promoter [67]. These findings could hint a possible regulatory involvement of oxidative stress and respiration in the regulation of *AOX* promoters.

In summary, the findings on both, *cis*- and *trans*-acting factors have revealed a complex regulation mechanism of P_{AOX1} , involving multiple factors. At least two activators, Mxr1p and Prm1p, were identified and one (Mxr1p) characterized in detail. Beside these direct activators, there was also evidence of a second level of

regulation. Mxr1p mediated activation was shown to be repressed by a 14-3-3 protein. However, because this effect was dependent on ethanol as carbon source, it seems possible that there are also alternate regulators taking over similar functions on other carbon sources. Also the exact molecular mechanisms of hexose transporter dependent repression of P_{AOX1} and also the function of Zta1p remain to be elucidated.

GAP promoter

The major advantages of P_{AOX1} driven expression are the strength and the tight regulation of this promoter. Especially for the production of toxic or detrimental heterologous proteins, the inducibility with methanol is favorable. Cultures can at first be grown to high cell densities under repressing conditions and in a second phase, heterologous protein production can be started by methanol induction [3]. However, this requires a two-step cultivation procedure, which increases the process time and handling efforts. For nontoxic proteins, which do not hamper cell growth, a strong constitutive expression system can help to minimize cultivation efforts and lead to a higher space/time yield.

Waterham *et al.* have characterized the *P. pastoris* glyceraldehyde 3-phosphate dehydrogenase gene (*GAP*) and showed that its promoter is strong and constitutive, reaching similar expression levels as P_{AOX1} [68]. Because of the constitutive expression, no induction step is needed which shortens and simplifies the process time for protein production and in some cases delivered similar and even higher product titers than P_{AOX1} driven expression. For these reasons, P_{GAP} has emerged as the most commonly used alternative to P_{AOX1} . Expression from P_{GAP} has been studied with respect to optimal vector design, the regulatory pattern and HCD bioreactor cultivations (reviewed by Zhang *et al.* [69]).

The *GAP* gene was initially isolated from a genomic library by colony hybridization with a probe of a *S. cerevisiae* *GAP* homolog. *GAP* is principally constitutively expressed, although there is a clear influence of the carbon source on the expression strength. Namely Northern blots showed, that the *GAP* mRNA levels were highest in cells grown on glucose and decreased approximately to two-thirds on glycerol, half on oleic acid and one-third on methanol [68]. Interestingly, the oxygen supply was also shown to affect P_{GAP} driven heterologous protein production [70]. Hypoxic conditions led to a three- to sixfold increased specific productivity in the production of three different but difficult to express heterologous proteins (a Fab fragment, human trypsinogen and porcine trypsinogen). Also the process times were decreased threefold and the volumetric productivity increased about twofold. In these studies *P. pastoris* was also shown to produce ethanol under hypoxic conditions (up to approximately 15 g/l), implying a change to oxidofermentative metabolism. The authors surmised that the hypoxic conditions could lead to a transcriptional upregulation of glycolysis genes like *GAP* [70]. However, these assumptions were not verified by the analysis of the transcript levels. Therefore it would also be possible, that the hypoxic conditions led to a weaker transcription by P_{GAP} , which could have been favorable for the expression of these difficult to express heterologous proteins.

Also in studies of *P. pastoris* alternative respiration, there were indications that P_{GAP} driven expression is not entirely constitutive but regulated under certain conditions [71]. The authors investigated the expression of a fusion protein of an alternative oxidase

and GFP under control of P_{GAP} . They noticed that the fluorescence increased upon depletion of glucose strongly for approximately two doubling times where transiently also some ethanol was produced. Yet the mechanism is unknown, and this effect was also noticed in other cases before [71].

In contrast to P_{AOX1} there is hardly any information available on *cis*-acting sequence elements or *trans*-acting factors involved in P_{GAP} regulation. Still, there have been efforts to optimize this already very strong promoter. Qin *et al.* took a random approach and created a library of P_{GAP} variants spanning a wide range of activities, which helped to optimize the expression of a methionine adenosyltransferase [72]. The starting point was a random mutagenesis of the P_{GAP} sequence by error-prone PCR and the screening of ~30,000 clones with a GFP variant as reporter gene. This resulted in P_{GAP} variants ranging from ~0.006- to 19.6-fold wild-type promoter activity. Seven variants, spanning the whole range, were further studied by determination of transcript levels by RT-qPCR and the use of two additional reporter enzymes (lacZ and the methionine adenosyltransferase). These strains were also confirmed to contain a single copy of the expression cassettes by qPCR. These results matched the initial results, the transcript levels were in some cases even more than 50-fold increased compared to the wild-type promoter [72]. It should be noted that these results are in a somewhat unclear relation to P_{AOX1} based findings. P_{AOX1} and P_{GAP} were considered to provide similar expression levels under the respective optimal growth conditions [68]. Published reports about P_{AOX1} optimization resulted however in a maximal 1.6-fold increase compared to the wild-type promoter [44,45] and not an almost 20-fold increase as in the case of P_{GAP} .

Alternative promoters

Despite the advantages of tightly regulated P_{AOX1} driven expression, the induction with methanol (a toxic and flammable compound) was considered problematic for safety reasons, especially for large-scale production [69]. Furthermore the strong expression from P_{AOX1} and P_{GAP} is not always the most favorable condition for heterologous protein production. If the correct folding of the heterologous protein or processing in the secretory pathway is the limiting step for high amounts of active product, a weaker expression level can be more desirable [3,73,74]. For example in the studies of P_{AOX1} variants, weaker and differently regulated variants showed higher yields of porcine trypsinogen than the wild-type promoter [47].

For these reasons, and also for the purpose of simultaneous expression of multiple different genes, there have also been efforts to identify alternative promoters with different regulatory properties and expression levels (Table 1 for an overview). In general these promoters are by far less well-studied than P_{AOX1} , and hardly any published research has been reported on *cis*-acting sequence elements or *trans*-acting factors involved in their regulation. Some promoters have already been discussed in previous review articles [3,69,75], therefore this section will focus on novel promoters. Information already available will only be shortly summarized and supplemented with new findings.

Promoters, which were already reviewed include P_{FLD1} (formaldehyde dehydrogenase), P_{PEX8} (peroxin 8), P_{YPT1} (a GTPase involved in secretion) [3], P_{AOX2} (alcohol oxidase 2) and P_{ICL1} (isocitrate lyase, ICL) [75].

TABLE 1

Promoters of *P. pastoris* genes used for heterologous protein expression. The approximate expression levels of alternative promoters relative to the classic promoters of AOX1 and GAP (listed at the top of the table) are given if they were provided in the respective reference. Promoters characterized by Stadlmayr *et al.* [93] were tested under different cultivation conditions with multiple reporter proteins, therefore the estimated expression range from weakest to highest is given

Gene name	Gene product	Regulation	Expression level	Reference
AOX1	Alcohol oxidase 1	Induced by methanol	Strong (naturally ~5% of mRNA and ~30% of total protein)	[3,21,27,29]
GAP	Glyceraldehyde 3-phosphate dehydrogenase	Constitutive	Strong (similar to P_{AOX1})	[68]
AOD	Alternative oxidase	Expression on glucose but not on methanol or upon glucose depletion if integrated in natural locus	~40% of P_{GAP}	[71]
AOX2	Alcohol oxidase 2	Induced by methanol	~5–10% of P_{AOX1}	[22,23]
DAS	Dihydroxyacetone synthase	Induced by methanol	Strong (similar to P_{AOX1})	[21,27,29,85]
ENO1	Enolase	Constitutive	~20–70% of P_{GAP}	[93]
FLD1	Formaldehyde dehydrogenase	Induced by methanol and methylamine	Strong (similar to P_{AOX1})	[76]
GPM1	Phosphoglycerate mutase	Constitutive	~15–40% of P_{GAP}	[93]
HSP82	Cytoplasmic chaperone (Hsp90 family)	Constitutive	~10–40% of P_{GAP}	[93]
ICL1	Isocitrate lyase	Derepression and ethanol induction	Not compared to P_{AOX1} or P_{GAP}	[83]
ILV5	Acetohydroxy acid isomeroreductase	Constitutive	~15% of P_{GAP}	[108]
KAR2	ER resident chaperone (also termed Bip)	Constitutive	~10–70% of P_{GAP}	[93]
KEX2	Endopeptidase involved in the processing of secreted proteins	Constitutive	~10% of P_{GAP}	[108]
PET9	ADP/ATP carrier of the inner mitochondrial membrane	Constitutive	~10–1700% of P_{GAP}	[93]
PEX8	Peroxisomal matrix protein	Induced by methanol or oleate	Weak	[3,79]
PGK1	Phosphoglycerate kinase	Constitutive	~10% of P_{GAP}	[92,93]
PHO89 or NSP	Sodium-coupled phosphate symporter	Induced by phosphate limitation	Strong (similar to P_{GAP})	[103,104]
SSA4	Heat shock protein	Constitutive	~10–25% of P_{GAP}	[93]
TEF1	Translation elongation factor 1 alpha	Constitutive and strong growth association	Strong (similar to P_{GAP})	[93,99,100]
THI11	Protein involved in thiamine biosynthesis	Completely repressed by thiamin	~70% of P_{GAP} on medium lacking thiamin	[93,111]
TPI1	Triose phosphate isomerase	Constitutive	~10–80% of P_{GAP}	[93]
YPT1	GTPase involved in secretion	Constitutive	Weak	[3,80]

P_{FLD1} was described to show similar strength as P_{AOX1} and also regulates the expression of a key enzyme of the MUT pathway [76]. However, in contrast to P_{AOX1} , P_{FLD1} is not only regulated by the carbon source but also the nitrogen source. This property was used to develop a methanol-free production process using methylamine for induction even in presence of glucose [77]. P_{FLD1} has been repeatedly used [78] and lately there were also commercial P_{FLD1} based vectors released (Life Technologies, Carlsbad, CA, USA).

P_{PEX8} and P_{YPT1} are weak promoters and considered alternatives in case P_{AOX1} or P_{GAP} driven expression is too strong, however they have only rarely been used [79,80]. Kranthi *et al.* identified five Mxr1p binding sites in the P_{PEX8} sequence [51]. Interestingly, the strong P_{AOX1} promoter contains only one site more, so the number of Mxr1p binding sites seems not to correlate with the promoter strength.

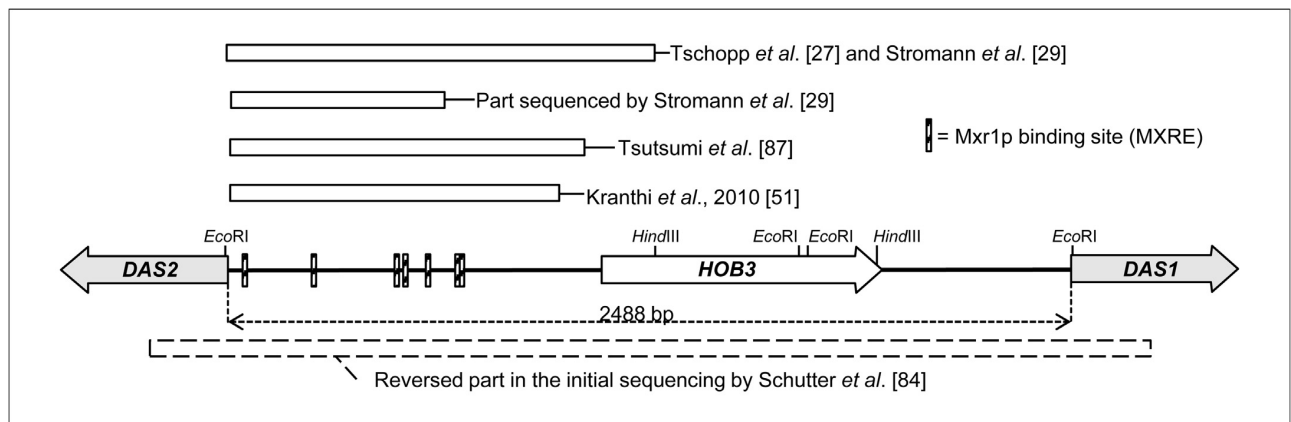
P_{AOX2} is providing about 10–20 times weaker expression than P_{AOX1} , but it is also methanol inducible and shows the same tight

regulatory profile as AOX1 [22,23,75]. P_{AOX2} expression was also optimized by adjusting the growth medium [81] and identifying regulatory elements [39,82] (see also section about P_{AOX1} regulation).

P_{ICL1} was used to express a dextranase and shown to be regulated on the transcriptional level by the carbon source by derepression and ethanol induction [83]. However, no comparison between P_{ICL1} and the benchmark promoters P_{AOX1} and P_{GAP} was performed. Therefore the regulatory features, strength and applicability of P_{ICL1} remained unclear [75].

DAS promoter

P_{DAS} is a promoter from the MUT pathway showing a similar regulatory pattern and expression level as P_{AOX1} [27]. In an early patent [29] and a related research article [27], only a single *DAS* gene was identified. In principal, this gene had even been previously reported in the screening of a cDNA library as an

**FIGURE 2**

Putative promoter regions between the *P. pastoris* *DAS1* and *DAS2* genes. The annotation of the CBS7435 strain was used [85]. Only the first 500 bp of the 2124 bp long *DAS* genes are shown, and the other elements are drawn to scale. *HOB3*, annotated as a probable guanine nucleotide exchange factor FLJ41603 homolog is also shown. The part that was reversed in the sequencing of the GS115 strain by De Schutter *et al.* [84] is approximately shown, based on Fig. 4 of Küberl *et al.* [85]. The *EcoRI*/*HindIII* fragment described by Tschoopp *et al.* [27] and Stromann *et al.* [29] is depicted. Also *EcoRI* and *HindIII* sites in the entire region are shown. The partial fragment sequenced by Stromann *et al.* is also shown. The *P_{DAS}* sequences used by Tsutsumi *et al.* [87] and Kranthi *et al.* [51] are also highlighted. Furthermore the Mxr1p binding sites identified by Kranthi *et al.* are also shown (the two most downstream binding sites are partially overlapping).

unidentified methanol regulated gene [21], but was not characterized as *DAS* until later [27]. Sequencing of the whole genome revealed, that there are two highly similar *DAS* genes in *P. pastoris*. The two genes are localized in the genome in close proximity to each other in reverse orientation, separated by only 2488 bp (Fig. 2) [84,85]. The two *DAS* genes show a high similarity of 91% [85]. In the first published sequence of the *P. pastoris* GS115 genome [84], the beginnings of the two *DAS* genes and the sequence between them were miss-annotated. Most probably due to the high sequence similarity of the two *DAS* genes the respective part was reversed. In a follow-up sequencing approach of the closely related wild type strain *P. pastoris* CBS7435, this issue was noticed and corrected (GenBank accession nos. CCA39320.1 and CCA39318) [85]. Notably the existence of the second *DAS1* gene was also described previously in a patent application and the related sequence is deposited in GenBank (accession no. FJ752551.1) [86]. Yet the very beginnings of the *DAS* genes and the upstream promoter sequence were also reversed in this sequence in a similar fashion as by De Schutter *et al.* [84]. Interestingly, there is also an additional gene annotated between the two *DAS* genes (*HOB3*, annotated as a probable guanine nucleotide exchange factor FLJ41603 homolog, GenBank accession no. CCA39319.1). The biological significance of this arrangement has however not been investigated. Research on the *DAS* promoter reported so far, has been performed using the promoter sequence of *DAS2*. Tschoopp *et al.* used an approximately 1350 bp *P_{DAS2}* fragment containing an *EcoRI*/*HindIII* fragment of *P_{DAS2}*, to drive the expression of a lacZ reporter gene [27]. This promoter was also patented and thereby partially sequenced (the respective sequences are shown in Fig. 2) [29]. Kranthi *et al.* identified seven Mxr1p binding sites in *P_{DAS2}* (Fig. 2) [51]. This is even one site more than in the *AOX1* promoter [48]. However, in contrast to *P_{AOX1}*, where the Mxr1p binding sites appear to be organized in tandem (Fig. 1), the binding sites in *P_{DAS2}* are rather dispersed over the whole promoter. There have also been efforts on optimizing *P_{DAS}*

by Tsutsumi *et al.* [87]. Similar to other studies on *P_{AOX}* they truncated the promoter length, performed internal deletions and added putative activating sequences. These variants were compared by using a phytase as reporter gene. For these studies also the sequence of *P_{DAS2}* (shown in Fig. 2) was used (GenBank accession no. GM709134). The authors did not mention the existence of two *DAS* genes and refer to the used promoter inconsistently as *P_{DAS1}*, indicating that they used the wrongly annotated genome sequence of the *P. pastoris* strain GS115. While the promoter sequence is the same in the different genome assemblies and annotations, the *DAS2* gene and not *DAS1* is transcribed from the reported promoter. The variants spanned 2–181% of the wild-type promoter activity and putative UASs were identified. These results were mainly achieved by focusing on an approximately 300 bp long internal fragment and the addition of putative UAS close to the 5' end. Studies of the putative TF Prm1p also indicated that it is involved in *P_{DAS2}* regulation [60]. The constitutive overexpression of Prm1p led to *P_{DAS2}* induction when grown on glucose (see also the section about *P_{AOX1}* *trans*-acting factors).

In contrast to *P_{DAS2}*, *P_{DAS1}* has so far only been little characterized in frame of an engineering study of the *P. pastoris* MUT pathway. Krainer *et al.* noticed that both genes were induced to the same extent upon MeOH addition [88].

PGK1 promoter

Phosphoglycerate kinase (*PGK*) is a constitutively expressed house keeping gene involved in glycolysis and gluconeogenesis. The individual native promoters of *PGK* have been widely used for the construction of expression vectors in several yeasts such as *S. cerevisiae* [89], *Yarrowia lipolytica* [90], *Candida maltosa* [91] and others [92]. *P. pastoris* *PGK1* was isolated by a PCR based approach. Conserved amino acid regions of homologs from other yeasts were identified and subsequently respective degenerate primers were designed. *PGK1* was then analyzed by a sequence-based approach for homologies to other yeasts, conserved domains, its codon

usage and regulatory *cis*-acting sequences in its promoter. The promoter was shown to be regulated moderately by the carbon source, as mRNA levels were two times higher on glucose than glycerol. P_{PGKI} was used to express an alpha-amylase but expression levels were only measured semiquantitatively and only in rough relation to P_{AOX1} [92]. Stadlmayr *et al.* characterized multiple novel *P. pastoris* promoters with three different reporter proteins [93]. In these studies P_{PGKI} showed consistently only 10% of P_{GAP} driven expression, indicating that P_{PGKI} is rather weak. Also deletion studies were performed. However, possible regulatory sequences have not been discussed and surprisingly a promoter variant which was truncated to only 250 bp was long enough to show full promoter strength [94].

TEF1 promoter

Translation elongation factor 1 alpha (Tef1) is a crucial component of the eukaryotic translation machinery and mediates the delivery of aminoacyl tRNAs to the ribosomes to sustain the elongation of the peptide chain [95]. The *TEF1* promoters from various fungi *Ashbya gossypii* [96], *Aspergillus oryzae* [97] and *Y. lipolytica* [98] show a strong constitutive expression suitable for heterologous protein production. The *P. pastoris TEF1* homolog was isolated by the use of degenerate primers of conserved regions and its promoter was studied in respect to regulation and expression levels [99,100]. Northern blots revealed a growth associated expression pattern of P_{TEF1} , resulting in high P_{TEF1} mRNA levels during exponential growth and clearly reduced levels in the stationary phase. P_{TEF1} driven expression was compared to P_{GAP} by using a lipase fused to a cellulose binding domain as reporter gene. The total activities measured from batch cultivation were similar for both promoters, however P_{TEF1} expression started earlier than P_{GAP} and showed a tighter coupling to the growth rate. In carbon-limited fed-batch cultivations the activity levels of P_{TEF1} driven expression exceeded P_{GAP} almost twofold. In studies by Stadlmayr *et al.*, P_{TEF1} was also found to be a strong promoter, but depending on the reporter protein and the cultivation time, the expression was varying between a similar strength as P_{GAP} or less [93]. Similar to the GAP promoter no specific *cis*- or *trans*-acting elements have been described for this *P. pastoris* promoter.

AOD promoter

Alternative respiration is a shortcut of the respiratory pathway that skips certain steps of the respiratory electron transfer chain [101,102]. Also in *P. pastoris*, an alternative oxidase (Aod) was identified and its significance for cell growth and viability was investigated [71]. In course of this study, the expression level of a fusion protein of the alternative oxidase and GFP was tested under control of the AOD promoter and the GAP promoter. Interestingly, P_{AOD} showed in the presence of glucose approximately the same fluorescence levels as P_{GAP} . However, expression from P_{AOD} started with a delay of about two to three hours. After reaching a peak at glucose depletion, the fluorescence levels of P_{AOD} driven expression decreased steadily to zero. By contrast, P_{GAP} fluorescence continuously increased and reached about twice the fluorescence of the P_{AOD} peak value. The authors argue that Aod is an unstable protein. This would suggest protein degradation as the cause for the fluorescence decrease in the P_{AOD} driven expression of the Aod-GFP fusion protein. A possible explanation would be that

expression from P_{AOD} ceases after glucose depletion, so the previously produced Aod-GFP fusion proteins get degraded. The constitutive GAP promoter would by contrast continue expression even upon glucose depletion, leading to the further fluorescence increase seen in the experiments. However, this explanation would require further proof by testing P_{AOD} and P_{GAP} with a stable reporter gene such as GFP alone. Interestingly P_{AOD} expression was also not induced by methanol, hinting a carbon source dependent regulation. The information currently available suggests that P_{AOD} is a strong promoter, reaching similar expression levels as P_{GAP} on glucose, but facing a repression or missing induction upon glucose depletion or presence of methanol [71]. On the one hand, these properties rather limit the achievable expression levels, as P_{AOD} driven expression stopped at about half of the maximal expression level of P_{GAP} . On the other hand this alternative regulatory profile might also be beneficial for the expression of certain proteins, as also some synthetic P_{AOX1} variants with lower expression levels and altered regulatory profiles led to higher yields than the wild-type promoter [47].

PHO89 promoter

P. pastoris PHO89 (also described as NPS promoter) was identified during a search for genes upregulated under phosphate-limited conditions and codes for a putative sodium-coupled phosphate symporter [103,104]. Phosphate-responsive promoters have been studied in other organisms as *E. coli* [105] and *S. cerevisiae* [106] and shown to be highly induced under phosphate-limited growth conditions. In an unrelated study, the *P. pastoris* acid phosphatase (*PHO1*) showed ~100-fold increased activity under phosphate limitation [107]. However, the transcriptional regulation of *PHO1* or the applicability for protein expression was not tested.

The putative promoter of *PHO89* showed a strong regulation by the phosphate concentration in the growth medium [103] by applying the same lipase reporter enzyme used for the studies of P_{TEF1} [99]. Under high phosphate concentrations no lipase activity was measured. Under reduced phosphate concentrations, similar activity levels as with P_{TEF1} or P_{GAP} were achieved. Notably the specific productivity (lipase activity/biomass/time) of the P_{PHO89} based expression process was 7.4 or 14.8 times higher than P_{TEF1} or P_{GAP} . The main reason was that the P_{PHO89} reached the maximal activity earlier and there was also less biomass formed than in the not phosphate-limited cultures of P_{TEF1} or P_{GAP} . The volumetric lipase activity from P_{PHO89} was however the same as P_{TEF1} and approximately twice of P_{GAP} . However, this combination of the reporter enzyme and growth parameters might not be representative for all conditions. P_{TEF1} driven expression was in similar experiments also twice as high as P_{GAP} [99], but with different reporter enzymes and cultivation conditions only about the same or less than P_{GAP} [93]. Hence, the patented P_{PHO89} driven expression appears to be a promising methanol-free system showing similar expression levels as P_{GAP} , but the maximum achievable expression levels remain to be verified with different reporter enzymes. Concerning *cis*-acting regulatory elements, the 1044 bp long promoter was also truncated, showing that a basic fragment of approximately 300 bp is able to mediate phosphate-responsive expression [104]. Furthermore, the authors also identified two putative binding sites for the TF Pho4p, which indicates that *PHO89* is regulated by the *PHO* regulatory system [103].

Other promoters which are less well studied include the *ILV5* and *KEX2* promoters of *P. pastoris* [108]. An initial characterization of the *ILV5* promoter showed that significant constitutive activity can be obtained using an approximately 1 kbp long region upstream of the *ILV5* gene. Also a truncated version of approximately 0.5 kbp was at least strong enough to drive the expression of selection marker genes in a new series of *P. pastoris* expression plasmids [109].

Postgenomic promoter studies

So far, all mentioned promoter studies were based on the isolation of the promoters by classic methods of genetics and molecular biology. This involved for example the search for homologs in other yeasts, then the design of degenerate primers binding to conserved regions and eventually the PCR amplification of a part of the CDS using these primers. Subsequently, the flanking region containing the promoter must be identified by inverse PCR [92,99,110]. Alternatively, to identify inducible promoters, mRNAs upregulated under certain growth conditions can also act as the starting point for the promoter search. This approach requires reverse transcription to cDNA and also the identification of the flanking regions [21,103]. However, sequencing of the *P. pastoris* genome and the first microarray experiments enabled a much simpler approach, as the promoters did not have to be isolated tediously by classic methods. Stadlmayr *et al.* identified 24 promising promoters from micro array data or by a literature search [93]. These promoters have also been reported as part of a patent [111]. The microarray data have been obtained by probing *S. cerevisiae* DNA microarrays with *P. pastoris* cDNA [112]. These heterologous microarrays were initially performed because *P. pastoris* DNA microarrays were not available at the time of this study. On the basis of the hybridization data of multiple cultivation conditions, the promoters of the 15 genes showing the highest expression levels were used for comparative studies [93]. Additionally nine promoters were rationally selected by searching the literature for strong and/or tightly regulated promoters from other yeasts.

Subsequently, the promoter sequences were obtained from a genome database (Integrated Genomics), then suitable primers were designed and the promoters were PCR-amplified from genomic DNA. All promoters were combined with three different reporter proteins including two intracellular proteins EGFP (enhanced GFP) and beta-galactosidase (lacZ) and the secreted protein HSA. Strains bearing the respective expression constructs were grown on different carbon sources (glucose, glycerol and methanol) and reporter levels were determined at multiple time-points. Notably also P_{AOX1} and P_{GAP} were included as references for strong methanol inducible and constitutive expression. In these experiments, 12 out of the 24 novel promoters showed constitutive expression under standard growth conditions in a full medium containing glucose. Few other promoters showed weak activity on glycerol or methanol, but the expression levels were less than 10% of P_{GAP} driven expression (promoters showing >10% activity of P_{GAP} are listed in Table 1). Notable constitutive promoters stem from glycolytic genes as *ENO1* or *TPI1* and genes involved in stress response like *HSP82*, *KAR2* and *SSA4*. Depending on growth conditions and the reporter used, these promoters were giving similar or weaker expression than P_{GAP} . None of the new

promoters were convincingly under multiple tested conditions stronger than P_{GAP} . Some novel promoters showed different regulatory profiles, like a stronger induction in the early or late growth phase, but reached similar total expression levels. The promoter of *THI11*, a gene involved in thiamin biosynthesis, showed the most remarkable regulation pattern. It was completely repressed on medium containing thiamin, but reached 70% of P_{GAP} on medium lacking thiamin. Therefore P_{THI11} driven expression could be used to set up a methanol-free production process, in which the starting point for expression could be simply set by the initial amount of thiamin in the medium.

An important issue about the study by Stadlmayr *et al.* is the copy number of the strains bearing the different promoter constructs. Multiple copies of a weak promoter can lead to the same expression as a single copy of a strong promoter [47], so this effect can strongly bias the results.

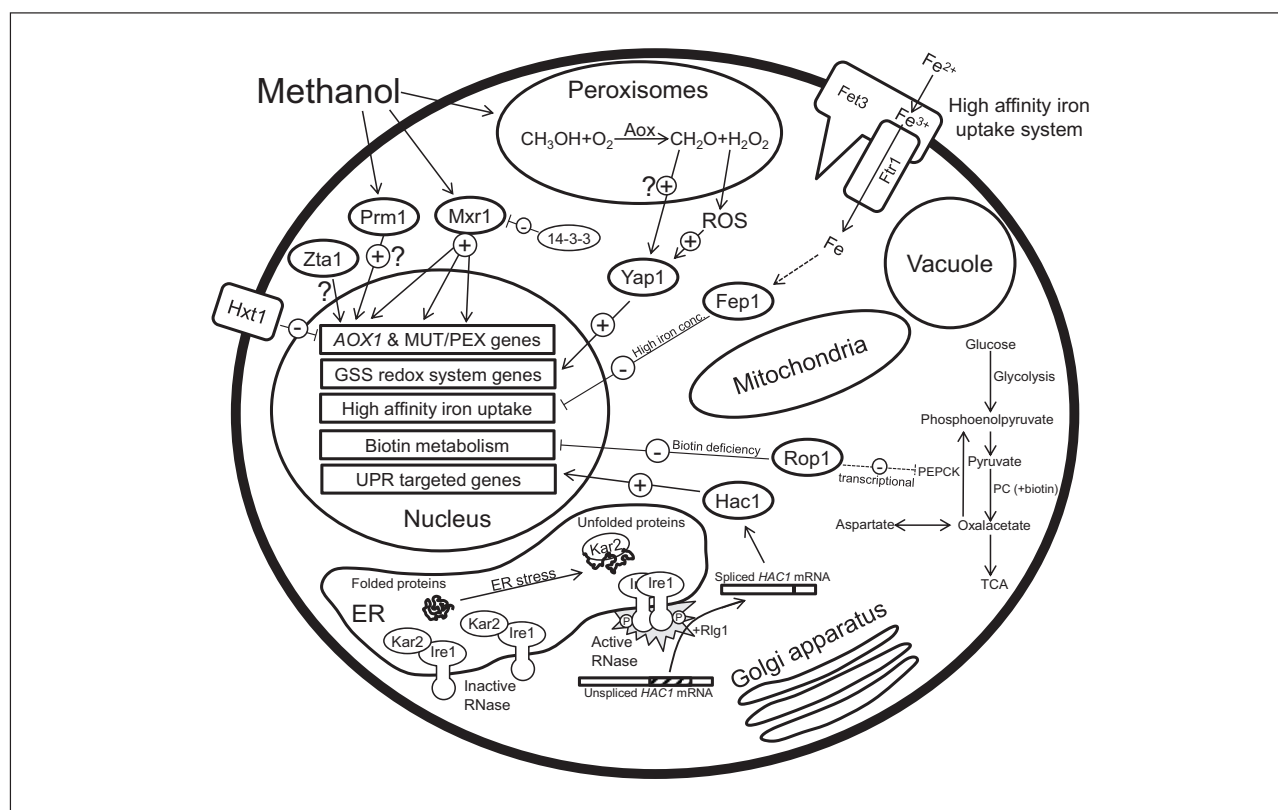
Stadlmayr *et al.* determined the copy numbers for four promoters by checking multiple transformants made with the same promoter construct. This revealed that 52% of the clones were single copy strains, 19% contained two copies, 13% three copies and the rest more than three copies. These results indicate a relative broad diversity. However as each promoter was tested with three different reporters and ten clones per reporter were tested, possible copy number effects might be compensated by the multiple analyses.

Indeed, the results of the three different reporter proteins were in general in accordance to each other, but in one case there was a drastic difference. P_{PET9} showed with EGFP a 10- to 17-fold higher fluorescence than P_{GAP} . Also the transcript levels were drastically increased, as shown by RT-qPCR measurements. This high expression was however not reproducible with the two other reporter enzymes HSA and lacZ with reporter activities of about only 10% of P_{GAP} . The authors did not find an explanation, but this example shows that the combination of promoter and protein of interest can have a strong influence on the expression levels. This observation is further underlined by a comparison of previous studies on P_{PGK1} [92] and P_{TEF1} [99] to the work by Stadlmayr *et al.* [93]. As mentioned above, P_{PGK1} was initially only assessed semiquantitatively and in approximate comparison to P_{AOX1} [92]. The thorough study by Stadlmayr *et al.* showed however clearly that P_{PGK1} reaches only about 10% of P_{GAP} driven expression. [93]. Similarly, P_{TEF1} driven expression was at first found to match or even twofold exceed P_{GAP} driven expression [99]. In the work by Stadlmayr *et al.*, P_{TEF1} driven expression never outperformed P_{GAP} and showed rather similar or lower expression levels [99].

Important transcriptional regulatory circuits

Introduction

Beside these sequence-based studies, there is a growing interest for regulatory networks and the TFs involved in the process of gene expression and protein folding. For example TFs involved in such diverse cellular processes as protein secretion (Hac1p), iron uptake (Fep1p), oxidative stress response (Yap1p) and biotin auxotrophy (Rop1p) have been studied in addition to the already discussed TF Mxr1p (Fig. 3 for an overview). The knowledge about the regulatory mechanisms enables TF-based engineering approaches and the design of new (synthetic) regulatory circuits to improve heterologous protein production processes.

**FIGURE 3**

Transcription factors and regulatory circuits involved in various cellular processes relevant for heterologous protein production in *P. pastoris*. The regulation of the AOX1 promoter by the *trans*-acting factors Mxr1p, Prm1p, Zta1p, Hxt1p and a 14-3-3 protein are shown. The oxidative stress response by Yap1p involved in ROS detoxification and methanol metabolism is depicted. Also regulation of the UPR by Hac1p (by splicing), regulation of the high-affinity iron uptake machinery by Fep1p and the role of Rop1p in biotin metabolism are shown. Positive regulatory effects are shown by a plus in a circle over the respective arrow. Negative regulatory effects are indicated by a minus in a circle and a truncated arrow. The schematic of the high-affinity iron uptake system is derived from Van Ho *et al.* [141], the schematic of the Rop1p regulated biochemical reactions are based on Kumar and Rangarajan [166] and the Hac1p regulation is based on Guerfal *et al.* [125] and Ma and Hendershot [132].

Regulation of the unfolded protein response by Hac1p

One of the main advantages of *P. pastoris* is that high titers of relatively pure secreted heterologous protein can be obtained on lab as well as on a large scale [113].

Gaining understanding of the secretory pathway is crucial for the identification of bottlenecks and improving the secretion of heterologous proteins further [114], for example, to obtain similar high yields as with filamentous fungi. Shortly summarized, secretory proteins need a signal sequence to enter the secretory pathway and are translated at the rough endoplasmic reticulum. Then they are transported from the ER lumen via the Golgi apparatus and secretory granules which fuse eventually with the plasma membrane [115]. The first step of translation at the rER, folding inside the ER and the introduction of posttranslational modifications are crucial steps in the standard secretory pathway. Unfolded or misfolded proteins in the ER cause cellular stress and activate the UPR pathway. The UPR upregulates chaperones to counteract the folding problems, but can also stimulate endoplasmic-reticulum-associated protein degradation (ERAD) or lead to programmed cell death (apoptosis) [116,117]. The overexpression of heterologous proteins is prone to activate the UPR, as the cellular machinery is not necessarily prepared to deal with the

processing and folding of large amounts of foreign proteins [73]. Therefore the UPR is an important factor to tweak protein secretion and has been studied extensively in *P. pastoris*. Initially the effect of the copy number, promoter and carbon source on the UPR were tested [74]. Furthermore the transcriptional regulation of genes targeted by the UPR was tested by TRAC (transcript analysis with aid of affinity capture) [118], a magnetic bead-based sandwich hybridization system [119] and microarrays [120]. The UPR was also found to participate in providing osmotolerance [121,122], to depend on the stability of the secreted protein [123] and to affect the cellular redox state [124].

However, the exact transcriptional regulation of the UPR in *P. pastoris* and the factors involved has only recently been characterized [125,126]. The general model of the transcriptional regulation of the UPR in yeast was at first elucidated in *S. cerevisiae* and found to be mediated by the TF Hac1p. Hac1p activates genes of the UPR and underlies a distinctive post-transcriptional regulation mechanism [127]. The *HAC1* mRNA contains an intron that blocks translation and needs to be spliced to permit translation of the functional protein [128]. Splicing of the *HAC1* mRNA is not accomplished conventionally by the spliceosome, but is linked by a unique mechanism to folding stress in the ER [129]. Namely

Ire1p, an ER resident transmembrane kinase/endoribonuclease, Kar2p (also named Bip), an ER-chaperon, and Rlg1p, a tRNA ligase, participate in *HAC1* mRNA splicing. Kar2p is involved in the regulation of Ire1p by binding to it under nonstress conditions [130,131]. If un- or misfolded proteins accumulate in the ER, they are bound by Kar2p, which assists proper folding. Thereby Kar2p releases Ire1p, which forms oligomers and is thereby autophosphorylated. Phosphorylated Ire1p activates its endoribonuclease activity and cleaves the intron from the *HAC1* mRNA. The mRNA is then religated by Rlg1p and functional Hac1p can be translated, migrate to the nucleus and bind to UPR responsive elements (UPRE) in the promoters of UPR target genes (Fig. 3 for a schematic representation) [117,132]. Notably the *HAC1* mRNA must also be transported from the nucleus to the Ire1p oligomers at the ER, to permit splicing [133].

The *P. pastoris* *HAC1* homolog was characterized recently and also found to contain the conserved intron known from *S. cerevisiae* and other fungi [125,126]. Interestingly, the significance of the splicing mechanism was somewhat inconsistent between two research groups. Guerfal *et al.* identified potential splice sites by sequence similarities with *S. cerevisiae* [125]. However, under various tested growth conditions no unspliced *HAC1* mRNA was detected, indicating a constitutive splicing of the *HAC1* mRNA in *P. pastoris*. The *HAC1* mRNA level increased strongly under conditions of folding stress, which would hint rather a transcriptional regulation mechanism of *HAC1* in *P. pastoris* [125]. By contrast, Whyteside *et al.* clearly detected the unspliced *HAC1* mRNA under unstressed conditions [126]. Under conditions of folding stress, the spliced form showed up, but the unspliced form was still detectable. Notably, both groups used the same *P. pastoris* same strain (GS115) and also the same agent in the same concentration to cause the folding stress, namely 5 mM dithiothreitol (DTT). Therefore the differences could possibly arise from different cultivation conditions and would have to be further tested to clarify the splicing of *HAC1* mRNA in *P. pastoris*.

Guerfal *et al.* tested furthermore the effect of *HAC1* co-expression on heterologous protein production [125]. There have been earlier attempts to co-express *HAC1* and the homolog of *S. cerevisiae* (SchAC1) in *P. pastoris* [118,119,134]. Constitutive expression of PpHAC1 showed little to no effect compared to the not co-expressing strain [125], while inducible co-expression using P_{AOX1} resulted in an up to twofold increased expression of surface displayed proteins, but in one case also, but in one case also a complete loss of the initial expression. Also the amount of correctly processed and folded membrane protein was increased.

In conclusion, characterization of PpHAC1 has shed light on the UPR in *P. pastoris* and engineering of the UPR by *HAC1* co-expression has led to increased yields and improved quality of heterologous proteins. Still, the exact circumstances of the *HAC1* mRNA splicing reaction in *P. pastoris* remain to be clarified. Also a general model for predicting the effect of PpHAC1 overexpression on the secretion efficiency of a certain heterologous protein would be favorable.

Regulation of iron uptake by Fep1p

Iron is a crucial cofactor of many proteins, as part of prosthetic groups like heme, iron-sulfur clusters or in nonheme iron proteins. Many biotechnologically relevant enzymes like peroxidases

and monooxygenases contain heme and the optimization of heme biosynthesis or addition of heme to the cultivations could significantly increase the yields of active enzyme [135,136]. Therefore the understanding of heme synthesis and iron uptake can be an important field for improvements in the production of heme or nonheme iron containing proteins and influence the general energy metabolism and productivity of the host. High-affinity iron uptake in *P. pastoris* has been found to involve similar machinery as in *S. cerevisiae* [137–139]. There are two main factors involved, the plasma membrane multicopper oxidase Fet3p and the iron permease Ftr1p [140,141]. Fet3p and Ftr1p interact and form a complex, in which Fet3p oxidizes Fe²⁺ to Fe³⁺, which can subsequently enter the cell via Ftr1p (Fig. 3 for a schematic overview). Depending on the availability of iron, the amounts of proteins involved in iron uptake and iron-dependent enzymes have to be controlled. In *S. cerevisiae* under iron scarcity, the high-affinity iron uptake machinery is upregulated [142–144], whereas at the same time entire pathways involving iron-dependent enzymes are repressed [145]. This regulation is exerted mainly on the transcriptional level, although also posttranscriptional mechanisms have been described [146,147].

In *P. pastoris*, Fet3p and Ftr1p mRNA levels increase under iron scarcity indicating a transcriptional regulation [138,139]. Miele *et al.* identified a TF, named Fep1 (Fe protein 1) that acts under iron abundance as a repressor of genes involved in high-affinity iron uptake [148]. The promoter sequence of Fet3p was analyzed for consensus sequences for GATA-type TFs, as these TFs are frequently involved in iron-dependent transcriptional regulation in other fungi [149–151]. Indeed, a putative GATA sequence element was found and mutated to test its regulatory role [148]. This P_{FET3} mutant affected the iron-dependent expression of a lacZ reporter gene. In detail, the wild type strain showed an increase of lacZ activity levels under iron scarcity, whereas the P_{FET3} mutant showed independently of the iron availability the same low lacZ activity. This supported the involvement of a GATA type TF in iron regulation in *P. pastoris*. On the basis of conserved regions of iron-responsive GATA factors from other fungi, degenerate primers were designed and a part of the putative FEP1 gene PCR amplified. A sequence comparison showed that Fep1p contains conserved Zn finger DNA-binding domains and a conserved motif of four cysteines that is typical of iron-responsive GATA TFs. A strain disrupted in *fep1* showed a constitutive expression of genes involved in high-affinity iron uptake independent of the iron concentrations in the medium. The wild type strain showed however strongly reduced expression levels of these genes under iron abundance. This indicates a repressing role of Fep1p, which is in accordance with the repressing function of Fep1p homologs from other fungi [149–151]. The authors showed furthermore that the DNA binding of Fep1p is dependent on the iron concentration [148]. In EMSA experiments, recombinantly produced Fep1p bound only in the presence of iron to the GATA sequence motif from the FET3 promoter. This further supports a model in which Fep1p represses genes for high iron affinity iron uptake under conditions of iron abundance.

Interestingly, despite the conserved machinery for high-affinity iron uptake between *P. pastoris* and *S. cerevisiae*, the transcriptional regulation mechanisms are different between these two organisms. In *P. pastoris* the genes are repressed by Fep1p

under conditions of iron abundance, whereas the same genes are activated in *S. cerevisiae* under iron starvation. *P. pastoris* is a respiratory yeast, whereas *S. cerevisiae* is a fermentative yeast, therefore the different response to iron could be a consequence of the different metabolic setups. This consideration is supported by the fact that also the respiratory yeast *S. pombe*, which is evolutionary only distantly related to *P. pastoris*, exhibits repression of high-affinity iron uptake genes in a similar Fep1p dependent fashion [148].

Oxidative stress response by Yap1p

Oxidative stress caused by reactive oxygen species (ROS) is a major factor of cellular stress and damages all cellular components such as proteins, nucleic acids and lipids [152–154]. ROS are caused by the high reactivity of oxygen and the nonspecific electron transfer by redox enzymes to unintended substrates. Consequently respiratory metabolism and the mitochondrial electron transport chain are especially notable sources of ROS in eukaryotes [155]. ROS is a general term not only referring to molecules such as H_2O_2 but also free radicals like the superoxide radical ($O_2^{\bullet-}$) or the hydroxyl radical ($OH^{\bullet}$) [156].

Cells contain a broad scope of defense mechanisms to counteract oxidative stress and ROS formation. This includes on the one hand antioxidants such as L-ascorbic acid (vitamin C), α -tocopherol (vitamin E), thioredoxin and glutathione (GSH). On the other hand this also includes enzymes like superoxide dismutase, catalase, peroxiredoxins and glutathione peroxidase protect cells against ROS [154].

Oxidative stress is also a relevant issue for recombinant protein production, as it decreases cell viability and triggers apoptosis [73]. Furthermore, ROS is involved in the UPR [124,157] and could therefore play a crucial role for stress associated with the industrially important protein secretion processes. ROS production is also an inevitable part of the methanol metabolism in methylotrophic yeasts, as the oxidation of methanol to formaldehyde by alcohol oxidase creates H_2O_2 as a side product (Fig. 3) [15]. Therefore the ROS detoxification plays a crucial role in the growth of methylotrophic yeasts and is tightly regulated.

In *P. pastoris*, the glutathione redox system was studied and found to be a crucial factor to permit growth on methanol [158,159]. Glutathione (γ -L-glutamyl-L-cysteinylglycine) is a non-ribosomal peptide and protects via its low redox potential against ROS [160]. In the process the reduced form of glutathione (GSH) is oxidized and forms a self-dimer via a disulfide bond between thiol groups of cysteine residues (GSSG). In *P. pastoris* oxidized glutathione is regenerated by reduction by a NADPH dependent glutathione reductase encoded by *GLR1* [158]. Other crucial genes involved in the glutathione redox system include glutathione peroxidase (*GPX1*), peroxiredoxin (*PMP20*), that act as peroxisomal glutathione peroxidase, and the enzyme catalyzing the rate-limiting step of glutathione synthesis (*GSH1*). Yano *et al.* showed that the glutathione redox system in *P. pastoris* is transcriptionally regulated by the TF Yap1p, which acts at the same time as a sensor for oxidative stress via the oxidation of conserved cysteine residues [158,159]. The *S. cerevisiae* Yap1p homolog has already been well characterized and shown to upregulate various genes involved in ROS defense [161,162]. ScYap1p contains a nuclear localization sequence, but is under normal growth conditions constantly

exported to the cytoplasm by a nuclear exportin. Under conditions of oxidative stress cysteine residues of ScYap1p are oxidized and form a disulfide bond. This causes a conformational change and ScYap1p is no longer recognized by the export machinery [163]. *P. pastoris* Yap1p was found to play a similar role in the oxidative stress response during the methanol metabolism. A *yap1* deletion strain did not show any phenotypical changes when cultivated under standard conditions on glucose, but growth on methanol caused a prolonged lag phase similar to a *gpx1* deletion strain [158]. The *yap1* deletion strain was also very susceptible to various ROS generating agents when grown on glucose [159]. Furthermore, PpYap1p showed a similar localization pattern as ScYap1p and localized to the nucleus under growth on methanol [158] and conditions of oxidative stress [159]. Consistently, the transcription of genes involved in the glutathione redox system, most notably *GPX1*, were upregulated under growth on methanol in the wild type strain, but not in the *yap1* deletion strain [158]. Similar to ScYap1p, PpYap1p is sensing oxidative stress via the oxidation state of conserved cysteine residues, which was tested by mutating the respective amino acid positions [159].

In contrast to these similarities to ScYap1p, PpYap1p was shown to play a special role in the methanol metabolism, namely the detoxification of formaldehyde by the Yap1p regulated GSH redox system [158]. GSH detoxifies formaldehyde by the spontaneous reaction to S-hydroxymethylglutathione [15]. Consistently, it was shown that cellular GSH pools increased in cells grown on methanol and also regeneration of GSSG to GSH was crucial. Namely, deletion of *GLR1*, responsible for GSH regeneration, led to hypersensitivity to methanol but also formaldehyde underlining the importance of the GSH redox system for formaldehyde detoxification. So besides the PpYap1p activation via the oxidation of conserved cysteine residues mentioned above [159], an activation by formaldehyde or GSH depletion was also suggested [158]. In conclusion this work showed the crucial role of PpYap1p for the MUT pathway and revealed a new role of Yap1p, as Yap1p homologs in other yeasts regulate only a stress response, whereas it is in *P. pastoris* crucial to sustain the metabolism of the carbon source methanol. Furthermore, overexpression or engineering of Yap1p might be a promising target to counteract oxidative stress arising from heterologous protein production.

Involvement of Rop1p in biotin metabolism

Biotin (vitamin H) is required by many carboxylases as an essential prosthetic group, but not all organisms are capable of a *de novo* biotin synthesis [164]. Notably, *P. pastoris* and also certain laboratory strains of *S. cerevisiae* are among the biotin auxotroph organisms [165]. Gasser *et al.* have engineered a biotin-prototrophic *P. pastoris* strain by reconstituting the biotin biosynthesis pathway by the expression of the respective genes from *S. cerevisiae* [165]. Kumar and Rangarajan have recently characterized a TF involved in the regulation of biotin metabolism related genes [166]. Initially they searched for proteins similar to Mxr1p, a key TF of MUT genes (see section about P_{AOX1} trans-acting factors), and identified a protein, later termed Rop, that shared 58% identity to the zinc finger domain of Mxr1p (in this review Rop is referred to as Rop1 to meet standard yeast naming conventions and to avoid confusion when referring to the protein). *ROP1* mRNA was only detectable under growth on methanol, but not on glucose, glycerol or oleic

acid suggesting a possible involvement in the regulation of MUT genes. However, this role could not be experimentally verified, as the deletion of *ROP1* neither affected the levels of the mRNAs of MUT genes nor the growth rate compared to the wild type on methanol. The authors compared the transcriptomes of the *rop1* deletion strain with the wild type by microarray and noticed an upregulation of genes involved in the biotin metabolism (e.g. biotin synthase, a biotin transporter and a biotin–apoprotein ligase) in the *rop1* deletion strain. This hinted a repression of biotin metabolism genes by Rop1p. Additionally, the *rop1* deletion strain was in contrast to the wild type able to grow on biotin deficient medium. The authors tried then to further elucidate the regulatory function of Rop1p and to find a biochemical explanation how the *rop1* deletion strain could grow under biotin deficient conditions [166]. This requires understanding of the role of biotin in the cellular metabolism (Fig. 3). One of the major biotin dependent enzymes is pyruvate carboxylase (PC), which converts pyruvate, the final product of glycolysis, to oxalacetate (OAA), a key intermediate of the tricarboxylic acid cycle (TCA). PC has been studied in *P. pastoris* by the identification of the gene and suppressor mutants [167], furthermore PC has also been shown to be involved in the assembly of Aox oligomers [168,169]. A PC deficient strain, caused by the lack of biotin or mutagenesis of PC, cannot replenish the OAA pool which stalls the TCA. However, the PC deficiency can be rescued by the addition of aspartate, which can be converted to OAA by transamination [170,171]. In case of *P. pastoris*, addition of aspartate to biotin deficient medium did restore the growth of the wild type strain, suggesting that the biotin dependent growth defect is caused by a lack of PC-dependent OAA synthesis [166]. The growth of the *rop1* deletion strain under biotin deficient conditions is therefore most probably permitted by a PC-independent pathway of OAA synthesis (PC bypass). These bypass reactions include ICL and malate synthase (MS) from the glyoxylate cycle and also phosphoenolpyruvate carboxykinase (PEPCK) [170,171]. In *P. pastoris* ICL and MS were expressed in the wild type and the *rop1* deletion strain grown under biotin deficient conditions. PEPCK was however only expressed in the *rop1* deletion strain and repressed in the wild type under biotin deficient conditions [166]. Rop1p is therefore involved in the repression of PEPCK under biotin- (and PC-) deficient growth conditions, therefore the name ‘repressor of PEPCK’ (ROP) was also chosen.

The authors also argued about putative binding sequences of Rop1p based on homologies of the zinc finger DNA-binding domain to Mxr1p. Furthermore, the transcriptional regulation exerted by Rop1p was additionally tested by comparing the *rop1* deletion strain and the wild type in a microarray experiment under biotin deficient conditions. The expression of several genes was clearly affected, but their exact physiological roles remain to be elucidated. It is also notable that the authors argue about the exact function of PEPCK sustaining growth on biotin deficient medium. PEPCK usually catalyzes the conversion of OAA to phosphoenolpyruvate (PEP), which is the first reaction of gluconeogenesis. However, the reaction can take place in the reverse direction and lead thereby to OAA and act as a PC bypass [170,171]. Yet, the glyoxylate cycle genes ICL and MS can also provide OAA in *P. pastoris*. Therefore PEPCK could also be growth limiting because of lack of PEP formation for gluconeogenesis and not because of

bypassing the biotin dependent PC reaction [166]. However, clarifying the exact role of PEPCK and thereby *ROP1* under biotin starvation will require further studies. Still, Kumar *et al.* found the first indication that a TF is involved in repression of PEPCK under biotin-deficiency and although not being the main focus of the study, they showed that a *rop1* deletion can grow under biotin deficient conditions. However, they did not compare the growth rate of the *rop1* deletion strain under biotin deficient conditions with the growth rate of the wild type on biotin. Therefore the biotechnological potential of using a *rop1* deletion strain for biotin free production processes remains unclear so far.

Conclusions

Recent research on promoters and the first partial knowledge about some transcriptional regulatory circuits in *P. pastoris* has shed light on the underlying molecular mechanisms and on how to improve heterologous protein production. The regulation of P_{AOX1} , the most commonly used promoter in *P. pastoris*, was studied in respect to *cis*-acting sequence elements and also *trans*-acting factors. Although its exact regulation is still unknown, at least various independent sequence-based approaches have identified putative regulatory sequences and TFBS. In part, the predicted putative TFBS were verified by the characterization of *trans*-acting factors and their recognition sites. Namely, two activators Mxr1p and Prm1p were identified. Mxr1p has been well characterized and shown to regulate MUT and peroxisome biogenesis genes. Furthermore the DNA-binding sites of Mxr1p have been identified and also a 14-3-3 protein interacting with Mxr1p has been described and shown to repress Mxr1p activity especially when grown on ethanol. However these findings indicate that there exist also additional, so far uncharacterized regulators of Mxr1p, that act in addition to the 14-3-3 protein on other carbon sources. Also other aspects of P_{AOX1} regulation are only partially understood. Namely the regulatory mechanism of Prm1p or the exact regulatory functions of hexose transporters and Zta1p remain to be elucidated. This indicates a highly complex regulation pattern of P_{AOX1} and other MUT genes, that involves multiple activating and repressing factors. Still, especially the sequence-based approaches have helped to further improve the already exceptionally strong *AOX1* promoter and also variants with altered regulatory profiles for applications in heterologous protein production were identified.

Beside this progress in understanding and improving P_{AOX1} , alternative promoters with different modes of regulation have also been identified. P_{AOX1} is providing extremely strong, controllable expression, but requires methanol, which is toxic and flammable, for induction. As an alternative to derepressed P_{AOX1} variants which do not rely on methanol anymore and can be simply regulated by changed carbon source feeding, especially strong and inducible promoters like P_{PHO89} or P_{THI1} could also become significant alternatives to P_{AOX1} , as they are regulated by the phosphate and thiamin concentration, respectively.

Furthermore, this review also covered TFs and their regulatory modes on diverse cellular functions such as UPR (Hac1p), iron uptake (Fep1p), oxidative stress response (Yap1p) and biotin auxotrophy (Rop1p). Overexpression of *HAC1* led in some cases to increased levels of secreted proteins and also elevated levels of correctly folded processed proteins. Yet, this effect was protein

dependent and also the regulation of *HAC1*, namely the significance of the splicing reaction, in *P. pastoris* remains to be clarified. Also, the regulatory circuits of Fep1p, Yap1p and Rop1p bear the potential to engineer the expression system *P. pastoris*, but have not been practically tested to improve bioprocesses so far. Namely, engineering of Fep1p mediated iron uptake could help to tweak the production of iron containing proteins. The regulatory role of Rop1p has hinted a bypass of biotin auxotrophy, which could help to design biotin independent *P. pastoris* strains. Oxidative stress is a known problem associated with heterologous protein production and arises especially from growth on methanol. Therefore improving the oxidative stress response by Yap1p could be an approach to design more robust production strains.

Although *P. pastoris* has only been used since approximately two decades by a broad public, significant progress has been made on characterizing key transcriptional circuits. The majority of molecular mechanisms investigated up to now, are still far from being completely understood. New technologies in nucleic acid sequencing, bioinformatics and high throughput analysis will hopefully provide a profound basis and stimulate further research on transcriptional regulation in *P. pastoris*.

Note added in proof

Just after this review was written, two research articles describing additional *trans*-acting factors involved in *P. pastoris* P_{AOX1} regulation have been published:

- Polupanov *et al.* investigated the role of a glucose sensor (termed GSS1) in autophagic degradation of peroxisomes and catabolite repression (Polupanov AS, Nazarko VY, Sibirny AA. Gss1 protein of the methylotrophic yeast *Pichia pastoris* is involved in glucose sensing, pexophagy and catabolite repression. *Int. J. Biochem. Cell Biol.* 2012;44:1906–18). A strain with a deleted *gss1* gene exhibited Aox activity on glucose containing

medium suggesting abolished glucose catabolite repression. Interestingly, ethanol catabolite repression was not affected in the *gss1* deletion strain. This supports previous findings that glucose and ethanol repression are exerted by different mechanisms (as discussed for the 14-3-3 protein [52] in the section “*Trans*-acting regulatory factors”).

- Kumar *et al.* have shown that Rop1p, previously described as a transcriptional repressor of genes involved in the biotin metabolism and as a repressor of PEPCK (see the section “Involvement of Rop1p in biotin metabolism”), is also repressing P_{AOX1} (Kumar NV, Rangarajan PN. The Zinc Finger Proteins Mxr1p and Repressor of Phosphoenolpyruvate Carboxykinase (ROP) Have the Same DNA Binding Specificity but Regulate Methanol Metabolism Antagonistically in *Pichia pastoris*. *J. Biol. Chem.* 2012;287:34465–73). Rop1p appears to act only on full media containing methanol as a repressor of P_{AOX1} and other MUT promoters by binding to the same binding sites as the main activator Mxr1p. Rop1p was shown to have a significantly higher DNA binding affinity than Mxr1p. This suggests a regulatory model, in which Rop1p and Mxr1p compete to regulate P_{AOX1} and other MUT promoters on full media in the presence of methanol. It is also notable, that Rop1p is regulating such diverse processes as biotin metabolism, methanol utilization and the expression of PEPCK.

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